nical
rary

SCIENTIFIC EDITION

MAR 5 1942

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

## CONTENTS OF THIS ISSUE

*Salvia Carnosa* (Dougl.). I—A Phytochemical Study . . . . . 33
*Allen I. White and Glenn L. Jenkins*

*Salvia Carnosa* (Dougl.). II—Carnosol . . . 37
*Allen I. White and Glenn L. Jenkins*

The Action of Some Organo-Mercurials and Some Copper Salts on Ringworm Fungi . 43
*Richard H. Barry, Walter H. Hartung and Thomas C. Grubb*

The Effect of Estrone and Stilbestrol on the Response of Rabbits' Uteri to Ergonovine 46
*Harry J. Wick and Clarence E. Powell*

Assay Methods for Phenothiazine Pharmaceuticals . . . . . 47
*Lewis E. Harris and Eloise B. Kerl*

The Utilization of Pigeons for the Biological Assay of *Adonis Vernalis*, N. F. VI . . . 49
*W. M. Benson and L. D. Edwards*

Germicidal Activity of Some Quaternary Ammonium Salts . . . . . 51
*H. G. Kollof, A. P. Wyss, R. E. Himelick and F. Mantele*

The Influence of Aloe and Podophyllum on the Flow of Hepatic Bile in the Dog . . . . 53
*Lloyd W. Hazleton*

The Assay of Yellow Mercuric Oxide Ointment, U. S. P. XI . . . . . 56
*F. N. Van Deripe and R. A. Konnerth*

Preliminary Antispasmodic Tests of a Series of Morpholino and Some Other Compounds . 57
*L. W. Rowe*

An Evaluation of the Cathartic Action of Bile Salts Following Acute and Chronic Administration to Mice . . . . . 60
*Lloyd W. Hazleton and Frank Fortunato*

Book Reviews . . . . . 64
Abstracts . . . . . 33–64
Advertising—Cover Pages 2, 3, 4, and pp. I to VI

ANNUAL CONVENTION—AMERICAN PHARMACEUTICAL ASSOCIATION
DENVER, COLORADO, AUGUST, 1942

Published by the American Pharmaceutical Association. Publication office: 20th and Northampton Sts., Easton, Pa. Editorial office: 2215 Constitution Ave., Washington, D. C.

Annual Subscription: Journal of the American Pharmaceutical Association, complete $5.00; Canada, $6.00; Other Foreign, $6.50. (*a*) Scientific Edition, $4.00; Canada, $4.35; Other Foreign, $4.50; Single Numbers, $0.35; Canada, $0.40; Other Foreign, $0.50. (*b*) Practical Pharmacy Edition, $1.50; Canada, $1.70; Other Foreign, $2.00; Single Numbers, $0.15; Canada, $0.20; Other Foreign, $0.25.

No claims will be allowed for Journals lost in the mails due to: Failure to notify of change of address (thirty days' notice required); after a lapse of three months; failure to receive wrappers from returned Journals.

Entered as second-class matter January 23, 1917, at the Post Office at Easton, Pa., under the act of March 3, 1879, as 24 times a year: Scientific Edition monthly on the fifth. Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

Vol. XXXI, No. 2 FEBRUARY, 1942

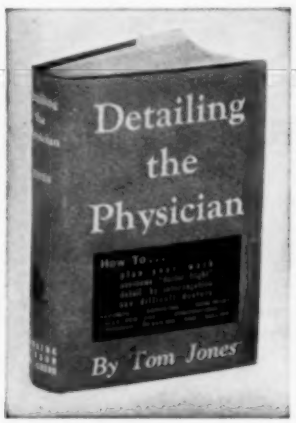
# USEFUL BOOKS FOR THE PHARMACIST

## DETAILING THE PHYSICIAN

*by Tom Jones*

**INCREASE YOUR SALES!**

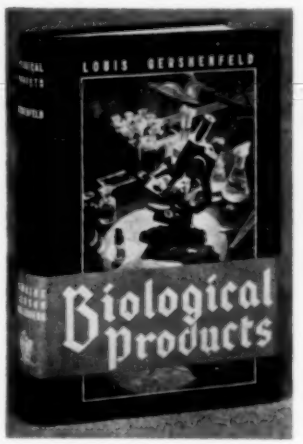
This single volume contains proven methods of productive detailing as practiced by nationally known manufacturers. An indispensable working tool for detail men, sales and advertising managers, pharmacists and surgical dealers.




**$2.75**

## BIOLOGICAL PRODUCTS

*by Dr. Louis Gershenfeld*




*290 Pages*

*40 Illustrations*

The first accurate, concise volume of complete information regarding the Biological Products. Indispensable for the physician, bacteriologist and public health worker.

**$4**

Send me.....BIOLOGICAL PRODUCTS

.....DETAILING THE PHYSICIAN OR BOTH

Enclosed check for $.....

**ROMAINE PIERSON PUBLISHERS, INC.**
**95 Nassau St. New York, N. Y.**

Name .....

Address .....



## APPLICATION FOR MEMBERSHIP

IN THE

# American Pharmaceutical Association

Approving the objects of the American Pharmaceutical Association, I hereby apply for membership in the Association and subscribe for the "Journal of the American Pharmaceutical Association." I enclose $ for my membership dues and subscription.

Check which you desire:

- ☐ Membership with the PRACTICAL PHARMACY EDITION, at $5.00.
- ☐ Membership with the SCIENTIFIC EDITION, at $6.00.
- ☐ Membership with BOTH EDITIONS, at $7.00.

Name in Full .....

*(Print name in full—Initials are not sufficient)*

Number and Street .....

Date..... Town..... State .....

Paid $..... No.....

This application with the first year's payment may be sent to the Chairman of the Membership Committee, the Secretary or any officer of the A. Ph. A.

**E. F. KELLY, Secretary,**
**2215 Constitution Ave.,**
**Washington, D. C.**

*Scientific Edition*

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXI | *FEBRUARY, 1942* | NUMBER 2 CONSECUTIVE NO. 3

## *Salvia Carnosa* (Dougl.). I—A Phytochemical Study*

*By Allen I. White† and Glenn L. Jenkins‡*

*Salvia carnosa* (Dougl.) is a North American plant of the Labiatae family found on the plains and slopes west of the Rocky Mountains from eastern Washington as far south as Nevada and California. It is a leafy, low, broad shrub, three-fourths to two and one-half feet high, bearing long spatulate or obovate, obtuse or retuse leaves. The bracts and upper floral leaves are tinged with rose or purple and the corolla is a deep blue. It is commonly called Purple Sage.

Taxonomists who questioned the classification of this plant in the genus *Salvia* have given it other botanical names (*Audibertia incana*, *Ramona incana*) but present-day taxonomists apply to it the name originally given by Douglas, that is, *Salvia carnosa*.

Attention was first directed to this plant because of its reputed use by the Indians as a medicinal herb in the treatment of colds, pneumonia, headache, stomachache, and for eyewashes (1). Heretofore, *Salvia carnosa* has received no attention as a possible source of drug material and no investigation of its character or chemistry has been found described in the literature.

The plant material used in this investigation was collected in the vicinity of Reno, Nevada, during the growing season of 1938, and was identified by W. Andrew Archer of the Division of Plant Exploration and Introduction, Bureau of Plant Industry, United States Department of Agriculture. The collection was divided into three portions. The first consisted of the entire overground plant, and preliminary investigation indicated that 50% alcoholic extracts possessed possible bacteriocidal activity.[1] This material was used in the general phytochemical studies. A second sample which also consisted of the entire overground plant did not possess appreciable bacteriological activity. It was not further

* Abstracted from a part of the thesis presented to the Graduate Faculty of the University of Minnesota by Allen I. White in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

This is a report in a coöperative research project connected with the Indian Medicinal Plant study of the Bureau of Plant Industry, United States Department of Agriculture.

Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.

† Instructor, School of Pharmacy, State College of Washington. Former Bureau of Plant Industry Agent.

‡ Dean, School of Pharmacy, Purdue University. Former Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

[1] The bacteriological tests were conducted by Dr. R. N. Bieter, Dr. W. P. Larson, Charles H. Drake and J. Gordon Beaton of the School of Medicine, University of Minnesota.

used in this investigation. The third sample consisted entirely of leaves and was used as a source of volatile oil. Investigation showed that the oil did not possess the characteristic bacteriological action of the extracts of the first sample. The object of this investigation was to make a preliminary phytochemical study of the whole plant material and especially to isolate and investigate the chemistry of the constituent responsible for the bacteriological activity of the extracts and the purported medicinal action obtained by the Indians.

## EXPERIMENTAL

The air-dried, dirt-free material was ground to about a No. 20 powder and well mixed. Using U. S. P. methods, it was found to contain 5.14 per cent total ash and 7.92 per cent of moisture.

*Preliminary Examination.*—The amount of volatile oil present in each of the three samples submitted for investigation was determined by the Clevenger method (2). The first overground sample contained 0.06%, the second contained 0.02%, and the leaves contained 3.46%.

One hundred grams of the powdered material were subjected to successive extractions with various solvents in a Soxhlet apparatus. The per cent of extractive was determined by evaporation of the solvent and drying of the residue at 100° C.

| Solvent | Per Cent |
|---|---|
| Petroleum benzin (b. p. 60–70° C.) | 2.40 |
| Diethyl ether | 0.70 |
| Chloroform | 0.70 |
| Ethanol | 4.50 |
| Ethyl acetate | 0.05 |
| Water | 3.80 |
| Total per cent of extractive | 12.15 |

The petroleum benzin extract was a reddish brown resinous residue. When it was dissolved in the smallest amount of petroleum benzin possible and cooled in a refrigerator for several hours, 0.45 Gm. of a light green wax precipitated out. It was purified by dissolving in a small amount of hot methanol and then cooling the solution and collecting the resulting amorphous precipitate. The diethyl ether, chloroform, alcohol and ethyl acetate extracts were brown, resinous residues. The water extract was brown in color, had a hard and brittle consistency, was practically odorless and had a slightly sweet taste.

Maceration and extraction of a portion of the plant material with Prollius' fluid failed to yield alkaloids as indicated by tests with the usual alkaloidal reagents. Examination for glycosides by a modified Stas-Otto method (3) gave negative results and glycosides were assumed to be absent.

### LARGE SCALE EXTRACTION

Four kilograms of the ground material were extracted by placing 1 Kg. at a time in a large glass percolator and extracting in the usual manner after a 24-hour maceration period with 50% aqueous ethanol. The procedure was allowed to continue until the percolate was nearly clear and no longer gave a test with ferric chloride test solution. The total extract from 1 Kg. of material amounted to about six liters.

*Isolation of Crystals.*—The extract so obtained was placed in a refrigerator and allowed to stand for two weeks. At the end of this period considerable crystalline material had collected and was removed by filtration. The filtrate was then replaced in the refrigerator and after standing for another two weeks, a second crop of crystals was collected. This procedure was repeated as often as necessary until no more crystalline material collected. Five or six collections were usually made. It was found by experiment that extreme cold or removal of ethanol would not facilitate the collection of the crystals without serious contamination. The crystalline material so obtained was readily purified by refluxing with charcoal in ethanol and after filtration, adding hot water to the hot solution to the point of incipient cloudiness and then allowing to cool gradually. Five recrystallizations from dilute alcohol produced clean, white, well-defined crystals. After drying for 24 hours over sulfuric acid in a desiccator, the total yield from 4 Kg. amounted to 25.2 Gm. or 0.63% of the total plant material. The material so obtained has been given the name "*Carnosol.*"

*Treatment of Extract with Acid.*—The slow separation of carnosol from 50% ethanol extracts suggested that it might be the product of enzyme action on a glycoside. To study the effect of acid upon the production of carnosol, sufficient hydrochloric acid to make a 0.5% solution was added to one liter of extract obtained in the usual way. This extract was shaken thoroughly and placed in a refrigerator with five other liters of extract. While the acid extract became slightly cloudy and deposited more impurities, no appreciable increase in rate of separation or amount of carnosol over the neutral extracts could be detected.

*Physical Examination of Crystals.*—Highly purified carnosol consists of white, odorless long needles which upon ignition burn without leaving a residue. After making sure the crystals were perfectly dry and free of any possible solvent of crystallization by drying for two hours at 78° C. and 2 mm. of pressure in an Abderhalden Drying Pistol containing phosphoric anhydride, the following physical constants were determined:

1. *Melting point:* The correct melting point of the compound was difficult to ascertain because it began to turn brown at about 190° C. and gradually softened until it melted with decomposition at 217° C. (uncorr.). When the melting-point tube was immersed in a bath at 215° C. and heated at the

rate of 1° every 20 seconds, the melting point was found to be 219.5° C. (uncorr.) with decomposition.

*2. Specific rotation:* When dissolved in dealdehyded ethanol and using a sodium flame and a 1-dm. tube in an ordinary polarimeter, the specific rotation of carnosol was found to be −66.00°.

*3. Solubility:* Carnosol is soluble in ethanol, ether, methanol and chloroform, and is slightly soluble in petroleum benzin. It is soluble with coloration in dilute alkali; alkaline solutions undergo a change from a beginning light orange-red to a deep brown to blue-black. The crystals were soluble also in concentrated sulfuric acid, producing a yellow color. Carnosol is insoluble in water, dilute hydrochloric acid and 5% sodium bicarbonate solution.

*Examination of 50% Ethanol Extract after Removal of Carnosol.*—When no more crystalline material could be isolated from the 50% ethanol extract, the solvent was removed by distillation under diminished pressure. The residue consisted of a dark brown resinous mass from which the water-soluble material was removed by the following method: To the residue in a 2-L. beaker on a steam bath were added 500 cc. of boiling water and the mixture stirred mechanically for one-half hour. The mixture was then allowed to cool and the insoluble material settled to the bottom of the beaker. The water-soluble fraction was then decanted off through a filter paper. This procedure was repeated two more times with 500-cc. portions of boiling water. The third extraction was very little colored. The water was removed from the combined aqueous extracts by distillation under diminished pressure. The light brown residue had a sweet taste and a very slight aromatic odor. The presence of carbohydrates in this extract was confirmed by the Molisch test (4). A small portion of the aqueous extract quickly reduced Fehling's solution, indicating the presence of reducing sugars. When a small amount of the extract was dissolved in water and a drop of ferric chloride test solution added, a deep blue-green color resulted which was probably due to the presence of tannins since bacteriological tests indicated that the residue was inactive. No further examination of this extract was made.

The portion of the 50% ethanol extract insoluble in water was a dark brown resin that possessed an aromatic odor. It was completely soluble in 95% ethanol. Since bacteriological tests indicated that the residue was inactive, no further examination was made of it.

*Examination of Marc of 50% Ethanol Extract.*—The marc remaining after the 50% ethanol extractive had been removed was subjected to the following examination: The material was packed in a large Soxhlet apparatus and extracted with petroleum benzin. The light green extract obtained deposited upon cooling a green wax similar to the wax isolated from the petroleum benzin extract mentioned in the discussion of extraction with successive solvents. Bacteriological tests showed the wax to be inactive. Very little other material was present in the petroleum benzin extract and it was not subjected to further observation.

The marc remaining after the extraction with petroleum benzin was then subjected to extraction with diethyl ether. The ether was removed by distillation and a small amount of a dark brown resin resulted. Bacteriological tests indicated that the extract was inactive. No crystalline material was obtained from it and it was not further examined. The total amount of resin extracted by 50% ethanol and by ether amounted to 6.3% of the crude plant material.

### VOLATILE OIL

Four and seventy-five hundredths kilograms of unground leaves were placed in a large still and steam distilled for six hours; 155 cc. of a volatile oil were collected from the distillate by mechanical separation and another 10 cc. were recovered from the aqueous portion by extracting it, after salting out, with petroleum benzin. The yield, weight to volume, was 3.46 per cent. Rectification by steam distillation produced 140 cc. of a clear, nearly colorless oil having an odor slightly resembling that of oil of eucalyptus. The rectified oil was dried by allowing it to stand over anhydrous sodium sulfate for 72 hours.

*Specific Gravity.*—The specific gravity as determined in a 10-cc. pycnometer fitted with a thermometer which dipped well into the liquid was 0.9209.

*Specific Rotation.*—The specific rotation of the volatile oil as determined in a polarimeter using a 1-dm. tube at 25° C. was +2.83°.

*Refractive Index.*—Using an Abbé refractometer, the average of five readings at room temperature was 1.4705.

*Solubility in Alcohol at 25° C.*—Five-tenth cubic centimeter of the volatile oil was placed in each of several graduated cylinders and alcohol of varying percentage strengths by volume was added until solution was just effected. The solubility is expressed as one volume of oil soluble or insoluble in a given volume of alcohol.

| Solvent | Solubility |
|---|---|
| 95% alcohol | 1 in 1 |
| 85% alcohol | 1 in 1 |
| 80% alcohol | 1 in 12 |
| 70% alcohol | 1 in 12 insoluble |

The 1 in 12 solution of 80% alcohol is just slightly acid to litmus.

*Saponification Value.*—This value was determined according to the method given in the U. S. P. XI (5). Sample A: 48.14; Sample B: 48.41. Average saponification value: 48.27.

*Acid Value.*—Using the method given in the U. S. P. XI (6), the acid value found was 1.62.

*Ester Value.*—The ester value as determined by difference between the saponification value and the acid value was 46.65.

*Acetyl Value.*—For this determination, the method suggested by Allan (7) was used. The total acetyl

Table I.—Volatile Oil of *Salvia carnosa* (Dougl.) Fractionation

| Fraction | Cc. | Boiling Point, °C. At 4 Mm. Pressure | Boiling Point, °C. At 730 Mm. Pressure | Refractive Index | Specific Gravity, 25° C. |
|---|---|---|---|---|---|
| 1 | 18.6 | 40– 49 | 169–170 | 1.4596 | 0.9001 |
| 2 | 3.3 | 58– 65 | 177–181 | 1.4611 | 0.9169 |
| 3 | 7.5 | 80– 85 | 195–197 | 1.4708 | 0.9437 |
| 4 | 4.0 | 90– 95 | Decomposition | 1.4750 | 0.9460 |
| 5 | 3.5 | 97–100 | Decomposition | 1.4828 | 0.9362 |
| 6 | 2.5 | 102–110 | Decomposition | 1.4903 | 0.9229 |
| 7 | 4.0 | 113–123 | Decomposition | 1.4952 | 0.9282 |
| Residue | 6.6 | | | 1.5142 | |

value was calculated to be 89.20 and the actual acetyl value determined by subtracting the ester value from the total acetyl value was found to be 42.55.

*Determination of Phenols.*—The method suggested by Allan (7) was used for this determination. The volume of unabsorbed oil from a 5-cc. sample amounted to 4.75 cc., showing 0.25 cc. or 5% of the oil had been absorbed. Since an alcoholic solution of the volatile oil gave no color reaction with ferric chloride test solution, it is probable that the value obtained was due to the solubility of some ingredients other than phenols.

*Investigation for Aldehydes and Ketones.*—An unsuccessful attempt to obtain a solid derivative when the volatile oil was treated with hydroxylamine hydrochloride indicated the absence of aldehydes and ketones.

*Fractionation of Volatile Oil.*—Fifty cubic centimeters of the oil were placed in a 250-cc. flask fitted with a Vigreux fractionating arm, water condenser and a graduated vacuum adapter. A diminished pressure of 4 mm. of mercury was furnished from a Hy-vac pump and heat was applied from an oil bath. Table I shows the result of the fractionation and certain physical constants of each fraction.

## DISCUSSION

In the experimental part of this paper, it was pointed out that the slow separation of carnosol from 50% ethanol extracts suggested the presence of a glycoside which upon enzyme hydrolysis yielded the crystalline product. While the presence of a glycoside could not be demonstrated by the Stas-Otto method, and the addition of acid to the extract did not increase the ordinary rate and amount of production of carnosol as might be expected if the production were dependent upon the hydrolysis of a glycoside, this is not sufficient evidence to disprove the glycoside theory. Many glycosides are soluble in 50% ethanol and the presence of a suitable hydrolyzing enzyme in such an extract is possible. However, enzymes are often inactivated by ethanol in concentration of 40–50% and higher. The possibility of a glycoside hydrolyzing during extraction must also be considered.

Since one sample of *Salvia carnosa* (Dougl.) which consisted of the entire overground plant yielded carnosol while another sample which also consisted of the overground plant did not, it appears that this compound may be formed at some particular stage in the growth of the plant. Further, since the sample which consisted of the leaves only was not a good source of the crystalline compound but was rich in volatile oil, while the overground samples did not contain an appreciable amount of volatile oil, it is possible that the crystalline compound is localized in the stems of *Salvia carnosa* and the volatile oil is localized in the leaves. These differences may also be due to varying local climate and soil conditions. Conclusive evidence in this matter can be derived only by further examination of the plant collected at known dates, during certain growth periods and at known places.

## SUMMARY AND CONCLUSIONS

1. The source and a description of *Salvia carnosa* (Dougl.) have been given.

2. The total ash and moisture content of the plant have been determined.

3. The amount and type of extractives yielded to various solvents on successive extractions have been determined.

4. Qualitative tests indicate that the plant contains carbohydrates, including reducing sugars, and tannins, but that it does not contain alkaloids.

5. Examination by the modified Stas-Otto method did not show the presence of a glycoside, but the presence of one is held possible.

6. A new crystalline compound possessing possible bacteriocidal activity has been

isolated and some of its physical properties determined. It has been given the name "Carnosol."

7. A wax and a resin have been isolated from extractives of the plant.

8. A volatile oil has been isolated by steam distillation of the leaves of *Salvia carnosa* (Dougl.). The usual physical and chemical constants have been determined and the oil separated by fractional distillation into eight fractions.

9. It has been established that the three lots of *Salvia carnosa* (Dougl.) examined differed greatly in their content of volatile oil and carnosol.

REFERENCES

(1) Henrichs, J. R., *Unpublished Observations* (1938).

(2) Clevenger, J. F., JOUR. A. PH. A., 17 (1928), 345.

(3) Rosenthaler, L., "The Chemical Investigation of Plants" (1930), G. Bell and Sons, Ltd., London, England, p. 22.

(4) Mulliken, "Identification of Pure Organic Compounds" (1911), John Wiley and Sons, New York, N. Y., Vol. I, p. 26.

(5) "United States Pharmacopœia XI" (1936), p. 445.

(6) *Ibid.*, p. 444.

(7) Allan, J., *et al.*, *Perfumery Essent. Oil Record*, 19 (1928), 131–132.

---

# *Salvia Carnosa* (Dougl.). II—Carnosol*

*By Allen I. White† and Glenn L. Jenkins‡*

In a previous paper (1) the authors reported the isolation of a new crystalline compound from *Salvia carnosa* (Dougl.). This compound, called carnosol, crystallizes in white, odorless, long needles and has a melting point of 219.5° C. (uncorr.) with decomposition. It has a specific rotation of −66.00° and is soluble in ethanol, methanol, ether and chloroform, and is slightly soluble in petroleum benzin. When placed in dilute alkali, carnosol dissolves and the solution undergoes a color change from a beginning orange-red to a deep brown to blue-black. It is also soluble in concentrated sulfuric acid, producing a yellow color. Carnosol is insoluble in water, dilute hydrochloric acid and a 5% solution of sodium bicarbonate.

This paper reports the investigations made concerning the chemical nature of this new compound which is of special interest because of the possibility that it is responsible for the bacteriological activity of extracts from which carnosol is obtained (1). Because of the insolubility in culture media of free carnosol and because of its apparent decomposition in alkali, it has been impossible to determine whether it has bacteriological activity or not.[1] It is hoped that an understanding of the chemical nature of carnosol will make it possible to prepare derivatives suitable for further bacteriological study.

## EXPERIMENTAL

*Reaction Tests.*—A 0.1% solution of carnosol in chloroform slowly and incompletely decolorized a few drops of a 1% solution of bromine in chloroform. However, when a 2% solution of potassium permanganate in acetone was added to a 1% solution of carnosol in acetone, rapid decoloration of the permanganate with production of considerable manganese dioxide occurred. When 0.1 Gm. of carnosol was dissolved in 5 cc. of hot ethanol and 0.1 Gm. of phenylhydrazine added, no cloudiness or precipitate formed upon cooling, dilution or standing. When a drop of ferric chloride test solution was added to a 0.1% solution of carnosol in ethanol, a deep brilliant green color was produced.

---

* Abstracted from a part of the thesis presented to the Graduate Faculty of the University of Minnesota by Allen I. White in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

This is a report in a coöperative research project connected with the Indian Medicinal Plant study of the Bureau of Plant Industry, United States Department of Agriculture.

† Instructor, School of Pharmacy, State College of Washington. Former Bureau of Plant Industry Agent.

‡ Dean, School of Pharmacy, Purdue University. Former Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

[1] The bacteriological tests were conducted by Dr. R. N. Bieter, Dr. W. P. Larson, Charles H. Drake and J. Gordon Beaton of the School of Medicine, University of Minnesota.

*Analysis.*—Using the usual sodium decomposition procedure, it was found that carnosol did not contain sulfur, nitrogen or halogens. Using the Rast camphor method, carnosol showed a molecular weight of 306.45. Calculated for $C_{19}H_{26}O_4$: 318.21. Ultimate analysis by the combustion method gave the following results: calculated for $C_{19}H_{26}O_4$: C = 71.65%, H = 8.24%; found: C = 71.90%, H = 8.07%. An alkoxyl determination employing the technique of Viebock and Schwappach as outlined by Clark (2) showed no alkoxyl group present. In an attempt to determine the saponification equivalent of carnosol it was found impossible to carry out the necessary titration and obtain an accurate result because of the reaction of the compound to alkaline solutions with the formation of a deep dark green to black color.

*Bromination.*—In a large test-tube, 0.5 Gm. of the crystals was dissolved in 20 cc. of hot glacial acetic acid and 1.5 cc. of liquid bromine were added. The mixture was allowed to stand for one hour during which no appreciable diminution in the intensity of the bromine color could be observed. The mixture was poured into 100 cc. of ice water and the resulting precipitate was filtered off. It was impossible to obtain a crystalline product from dilute ethanol, but by dissolving the precipitate in a small amount of warm glacial acetic acid and diluting with warm water to the point of incipient cloudiness, crystals were obtained. They were further purified by crystallization from anhydrous ether several times. A very small yield of well-defined, rectangular, greenish brown crystals was obtained. Melting point, 189–190° C. (uncorr.).

*Hydrogenation.*—In a pressure bottle, 0.2 Gm. of carnosol was dissolved in 30 cc. of absolute ethanol and a small amount of platinum black was washed in with an additional 20 cc. of absolute ethanol. The mixture was shaken with hydrogen for one hour at room temperature under a pressure of 41 lbs. per sq. in. The catalyst was filtered off and the solution concentrated to 20 cc., warm water added to the point of incipient cloudiness, and the material allowed to crystallize. Melting point, 219–220° C. (uncorr.) with decomposition. Browning began at 190° C. Mixed melting point with original crystals, 219° C. (uncorr.) with decomposition. Conclusion: No hydrogenation took place.

*Methylation.*—In a 250-cc. Erlenmeyer flask, a dry ethereal solution of diazomethane prepared from 6.9 Gm. of *n*-nitroso methyl urea (3) was added to one-hundredth of a mole (3.18 Gm.) of the crystals dissolved in a mixture of 50 cc. of anhydrous ether and 20 cc. of anhydrous methanol cooled in an ice bath to 0° C. The flask was fitted with a calcium chloride tube, and after one hour in the ice bath it was kept at 5° C. in a refrigerator for 48 hours. At the beginning of the reaction, considerable escaping gas was observed but quickly decreased to a slow evolution. At the end of the period, the solution was poured into an evaporating dish and allowed to evaporate spontaneously to approximately 15 cc. It was then cooled in an ice-salt bath, crystals forming. Four recrystallizations from dilute ethanol produced white platelets having a melting point of 152.5–153.5° C. (uncorr.). Yield, 1.4 Gm. (40.58%). The compound was soluble in ethanol, methanol and ether. It was insoluble in water and 10% sodium hydroxide. An alcoholic solution gave no color reaction when treated with ferric chloride test solution. Calculated for $C_{21}H_{30}O_4$: C = 72.78%, H = 8.73%; found: C = 72.98%, H = 9.08%. Methoxyl determination (2) of methylated carnosol showed the following results: calculated for $C_{21}H_{30}O_4$ (two $OCH_3$): 17.92%; found: 17.94%. Calculation of molecular weight from the per cent of radical found: 345.82; calculated for $C_{21}H_{30}O_4$: 346.24. By calculation, the molecular weight of carnosol would be 317.79. From these molecular weight determinations and the ultimate analyses, it is justifiable to assign the molecular formula of $C_{19}H_{26}O_4$ to carnosol and $C_{21}H_{30}O_4$ to its methylated derivative.

*Saponification of Methylated Carnosol.*—Since the difficulty encountered in saponification of carnosol was believed due to phenolic groups present, saponification of the methylated derivative was undertaken in the belief that this difficulty could here be avoided. The saponification equivalent as determined using an ethanolic sodium hydroxide solution was 334.41. Calculated for $C_{21}H_{30}O_4$: 346.24. The reaction mixture was then examined for an acidic saponification product as follows: The solution was made alkaline to phenolphthalein with dilute sodium hydroxide, and after the addition of 10 cc. of water, the ethanol was evaporated from the solution on a steam bath. The solution was then made acid by the addition of a few drops of dilute hydrochloric acid and a flocculent white precipitate resulted. It was removed by filtration and washed with ice water until the filtrate was acid-free to litmus. Attempts to crystallize this amorphous precipitate from dilute ethanol, benzol-petroleum benzin, dilute methanol and ether were unsuccessful. The potassium salt of the acid was readily and successfully crystallized by making an alcoholic solution of the acid alkaline with dilute potassium hydroxide and cooling the resulting solution. Fine white needles resulted. The amorphous precipitate of the free acid melted over a range of 140–160° C. The yield was 70 mg. An alcoholic solution gave no color reaction when treated with ferric chloride test solution.

An attempt to acetylate 0.1 Gm. of methylated carnosol using 2 cc. of acetic anhydride with 0.05 Gm. of fused sodium acetate was unsuccessful, indicating the methylated compound contained no free OH group.

*Acetylation.*—One gram of carnosol was mixed with 0.5 Gm. of powdered fused sodium acetate and 5 cc. of acetic anhydride and refluxed on a steam bath, with occasional shaking for two hours. The hot solution was then poured into ice water and allowed to stand in a refrigerator for four hours.

The solid material which had formed was collected and recrystallized four times from dilute ethanol. After drying for 24 hours in a sulfuric acid desiccator, the melting point was 165–166° C. (uncor.). The yield was 1.03 Gm. (81.7%). An alcoholic solution gave no color reaction when treated with ferric chloride test solution. Calculated for $C_{32}H_{30}O_6$: C = 68.61%, H = 7.52%; found: C = 68.37%, H = 7.84%.

An acetyl determination was made in the following manner: Exactly 0.0992 Gm. of the compound was placed in a 250-cc. round-bottom flask and 25 cc. of approximately 0.1*N* alcoholic potassium hydroxide were added. The solution was refluxed for one hour over a small flame. The solution was then cooled and made acid with dilute sulfuric acid. Sufficient water was then added to make a volume of approximately 200 cc. The solution was carefully distilled over a low flame into exactly 50 cc. of 0.02*N* sodium hydroxide until approximately 50 cc. remained in the distillation flask. During the distillation, the adaptor dipped slightly into the sodium hydroxide solution. The sodium hydroxide solution was then titrated using a micro burette with 0.02*N* hydrochloric acid, using phenolphthalein as the indicator. Calculated for two $CH_3CO$ groups in $C_{32}H_{30}O_6$: 21.38%; found: 20.81%.

*Benzoylation.*—One gram of carnosol was dissolved in 3 cc. of pyridine and 1 cc. of benzoyl chloride added. The solution was refluxed for one-half hour over a small flame after which it was poured with vigorous stirring into 10 cc. of ice water. The precipitate which formed was washed with 5 cc. of a 5% sodium carbonate solution and recrystallized four times from dilute ethanol. After drying for 24 hours in a sulfuric acid desiccator, the melting point was 172° C. (uncorr.). Yield, 1.44 Gm. or 87.27%. An alcoholic solution gave no color reaction when treated with ferric chloride test solution. Calculated for $C_{32}H_{24}O_6$: C = 75.25%, H = 6.51%; found: C = 75.20%, H = 6.87%.

*Vacuum Sublimation.*—Seventeen-hundredths gram of carnosol was subjected to vacuum sublimation for four hours at a temperature of 200° C. and a partial pressure of 3 mm. of mercury. In that period of time, 0.06 Gm. of an amorphous sublimate was collected. It had a light tan color mixed with white, indicating some decomposition. Recrystallization of the sublimate from dilute ethanol gave crystals having a melting point of 219° C. (uncorr.) and which caused no depression of the melting point when mixed with carnosol. An alcoholic solution gave the characteristic green color reaction when treated with ferric chloride test solution. The unsublimed residue amounted to 0.10 Gm. and tests showed it to be unchanged carnosol. Thus, carnosol is slowly sublimable under reduced pressure with some slight decomposition.

*Saponification.*—One gram of carnosol was refluxed for six hours with 25 cc. of 25% aqueous potassium hydroxide. The alkaline solution was then carefully and slowly distilled until 5 cc. of distillate had been collected. Difficulty was encountered in the distillation due to excessive frothing. The distillate was divided into two portions, one of 2 cc. and one of 3 cc.

The 2-cc. portion was used for an attempted fractionation on a semi-micro scale according to the procedure of Emich (4). Three fractions were obtained and gave the following boiling points at 730-mm. pressure using the procedure given by Emich (4).

| Fraction | B. P., ° C. |
|---|---|
| 1 | 83.0 |
| 2 | 91.5 |
| 3 | 93.0 |

The 3-cc. portion was used in an unsuccessful attempt to make a derivative with 3,5-dinitrobenzoyl chloride.

The alkaline reaction mixture which remained after distillation was examined by extracting with ether while alkaline and then making acid and extracting with ether. Both extractions produced uncrystallizable resinous extracts which gave green colors characteristic of carnosol when tested in ethanolic solutions with ferric chloride test solution. The presence of an alcohol with a boiling point below 100° C. as the neutral saponification product is indicated but not confirmed.

*Permanganate Oxidation.—1. In neutral acetone:* The procedure as outlined by Adams, Hunt and Clark (5) for the oxidation of tetrahydrocannibidiol was adopted. In a three-necked one-liter flask equipped with a mechanical stirrer, dropping funnel and reflux condenser, a solution of 5 Gm. of potassium permanganate in 250 cc. of acetone was added over a two-hour period to 1 Gm. of carnosol in 50 cc. of acetone. The reaction was begun at room temperature but at the end of 30 minutes a small flame was applied to speed up the reaction. At the end of this period, the solution retained the characteristic permanganate color. The solution was made acid with sulfuric acid and the excess manganese dioxide and permanganate were destroyed by the addition of sodium bisulfite. The clear, pale yellow solution was diluted with 50 cc. of water and the acetone distilled off. The remaining aqueous solution was extracted with three successive 25-cc. portions of ether and the ether solution in turn extracted with two successive 15-cc. portions of 5% sodium bicarbonate solution. The bicarbonate layer was acidified with sulfuric acid, a slight cloudiness occurring. The acidified solution was extracted with ether and the ethereal solution dried for 12 hours over anhydrous sodium sulfate. Spontaneous evaporation of the ethereal solution produced a light brown residue having a pungent, acrid odor. Yield, 0.24 Gm. The ether solution containing the sodium bicarbonate insoluble material was washed twice with water, passed through a dry filter, and allowed to evaporate spontaneously. A reddish brown residue having a slight aromatic odor but giving no color reaction with ferric chloride solution was deposited. Yield, 0.47 Gm. Attempts to crystallize both fragments

were unsuccessful. Attempts to prepare a solid derivative with *p*-nitro benzyl bromide according to the procedure of Lyons and Reid (6) and an anilide according to the procedure outlined by Kamm (7) of the sodium bicarbonate fraction were unsuccessful.

*2. Alkaline oxidation in water:* To 1 Gm. of carnosol in a three-necked one-liter flask equipped with a mechanical stirrer, reflux condenser and dropping funnel, was added gradually a 2% aqueous solution of potassium permanganate and the solution boiled over a low flame. After the addition of the first 25 cc. of the permanganate solution, 10 cc. of 10% sodium hydroxide solution were added. The addition of the permanganate solution was then continued at such a speed that the solution maintained its characteristic color. At the end of 60 minutes, approximately 6 Gm. of potassium permanganate had been consumed and the solution did not decolorize. After cooling, the manganese dioxide formed was removed and washed on a Büchner funnel and the filtrate concentrated to 10 cc. The solution was then made acid with 10% sulfuric acid, a slight cloudiness occurring. No product could be obtained from this solution even when cooled to −20° C. in an ice-salt bath. The solution had a strong, disagreeable odor.

The manganese dioxide precipitate was washed with four 20-cc. portions of ether which were combined and evaporated to dryness. A small amount of a yellowish green oil which could not be crystallized was obtained.

*Zinc Dust Distillation.*—Using the procedure of Clar (8), the following distillation was carried out: In a 50-cc. Pyrex distilling flask equipped with a mechanical stirrer and air condenser and resting in a Wood's metal bath, 3 Gm. of carnosol, 3 Gm. of sodium chloride, 15 Gm. of fused zinc chloride and 10 Gm. of zinc dust were brought to fusion. The temperature of the bath was slowly raised from 220° to 320° C. as a yellow, oily distillate having a light blue fluorescence was collected. The distillate was washed from the condenser by means of a small amount of anhydrous ether and the ether evaporated. Yield, 1.25 Gm. A dilute alcoholic solution of the distillate had a beautiful blue fluorescence and did not give any color reaction when treated with ferric chloride test solution. It could not be crystallized, even at low temperatures, from ethanol, dilute ethanol or ether, but was purified and dried by distillation over sodium. It was found to be soluble in ethanol, ether and chloroform, and insoluble in water, sodium hydroxide, dilute hydrochloric acid and concentrated sulfuric acid. When 0.05 Gm. of the distillate was dissolved in 2 cc. of chloroform and a drop of bromine in chloroform was added the bromine was not decolorized in five minutes. When 0.05 Gm. was dissolved in 2 cc. of acetone and 2 drops of a 2% solution of potassium permanganate were added, a slight but slow decoloration of the solution occurred at the end of three minutes. An attempt to make a picrate using the procedure suggested by Kamm (9) was unsuccessful. Boiling point, 298–300° C. $n_D^{25} = 1.5584$. Calculated for $C_{14}H_{18}$: C = 90.26%, H = 9.74%; found: C = 90.08%, H = 9.50%.

*Sulfur Dehydrogenation of the Zinc Dust Distillate.*—Dehydrogenation of the zinc dust distillate was carried out in the following manner: Twenty-five hundredths gram of the distillate and 0.16 Gm. of sulfur were put in a small Pyrex tube and a Pyrex air condenser fused onto it. The mixture was heated for six hours at 140° C. and then for 18 hours at 180° C. in an oil bath. Hydrogen sulfide was continuously evolved during that period. After allowing to cool, the Pyrex tube containing the black reaction mixture was crushed and the residue extracted with ether. This solution, which showed a pale blue fluorescence, produced a small amount of crystalline material upon evaporation. Recrystallization five times from small amounts of ethanol gave a product (0.06 Gm.) having a melting point of 97° C. A mixed melting point with a known sample of phenanthrene (m. p., 97.5–98.5° C.) showed no depression. A picrate made in alcoholic solution produced orange-red crystals which after drying at 100° C. for 15 minutes gave a melting point of 140° C. A mixed melting point with the picrate of a known sample of phenanthrene (m. p., 141° C.) showed no depression. From these results it is concluded that the sulfur dehydrogenation product of the zinc dust distillation of carnosol is phenanthrene.

Dehydrogenation in a similar manner using selenium metal in place of sulfur produced too small an amount of crystalline material for characterization.

*Pyrolysis.*—To determine whether the zinc dust distillate obtained above might be a product of simple pyrolysis of carnosol, the following experiment was carried out: In a small Pyrex tube to which had been attached a distilling arm, 0.2 Gm. of carnosol was heated with a naked flame from a micro burner. The compound melted and changed into a red liquid which bubbled rapidly as the heating continued. A light red oil distilled and, upon cooling, changed into a viscous and stringy resin. A small carbonaceous residue remained in the distilling flask.

The resin-like distillate was dissolved with some difficulty in a small amount of boiling ethanol. A small portion of the solution gave no color test with ferric chloride test solution. To the remainder of the warm solution warm water was added to the point of incipient cloudiness and then placed in the refrigerator over night. A small amount of crystalline material was collected and purified by boiling with charcoal in alcohol and recrystallizing twice from dilute ethanol. The white needle crystals obtained melted at 160–162° C. (uncorr.). They were insoluble in 10% sodium hydroxide but soluble in concentrated sulfuric acid, causing a deep yellow coloration. No further investigation of their nature was made.

Since it was considered possible that the gas given off when carnosol was liquefied might be carbon dioxide, the following experiment was performed: 50

mg. of carnosol were placed in a porcelain boat inside a Pyrex tube, one end of which was connected to an oxygen tank; the other end was fitted with a small glass tube which dipped into a solution of calcium hydroxide. The air was first removed from the tube by passing through oxygen until the lime water showed no precipitation. Then the tube was dipped into a fresh solution of calcium hydroxide and, while continuing the passage of oxygen through the tube, the boat was slowly heated until the crystals melted and then held at that temperature while the resulting liquid bubbled. The flow of oxygen was continued through the tube for five minutes, but no cloudiness could be observed in the solution of calcium hydroxide. Thus, the gas given off by the pyrolysis of carnosol is not carbon dioxide.

## DISCUSSION

The new organic compound, carnosol, which had been isolated from *Salvia carnosa* (Dougl.) has been assigned the molecular formula, $C_{19}H_{26}O_4$. The molecular weight as determined by the Rast method and the methoxyl determination of the methylated derivative is satisfied by this formula. The results obtained by ultimate analysis of the original compound as well as of the methylated, acetylated and benzoylated derivatives also confirmed this formula.

The presence of phenolic OH groups in carnosol was suggested by the reaction of the compound to ferric chloride test solution and its solubility and oxidation in sodium hyroxide. Methylation of carnosol with diazomethane and the determination of the methoxyl added indicated that two of the four oxygens in the compound exist in phenolic OH groups. The presence of two OH groups was confirmed by the acetyl determination of the acetylated compound. That the two OH groups acetylated are the same as the two methylated was shown by the fact that the methylated compound could not be acetylated. Although alcoholic OH groups are acetylated as well as phenolic OH groups, diazomethane does not methylate alcoholic OH groups but readily methylates phenolic OH groups. Since a methoxyl determination on the unmethylated carnosol showed that it contained no methoxyl group, the value obtained from the methylated compound must be due only to groups added by diazomethane. From these facts it was concluded that the two oxygens present in OH groups are both phenolic OH groups.

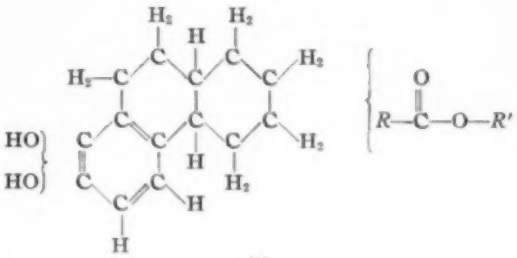
Because of the oxidation of carnosol in alkaline solutions, saponification and acidity determinations could not be made on it. However, the fact that the compound was insoluble in 5% sodium bicarbonate solution indicated that no free carboxyl group was present. This was further borne out by the insolubility of the methylated compound in sodium hydroxide. The determination of the saponification equivalent of the methylated compound demonstrated the presence of an ester. This ester could be the result of methylation of a carboxyl group by diazomethane. A methyl ester and one methoxyl group would give the results obtained in the methoxyl determination. However, if this were so, the diacetate which has been shown to be formed by acetylation of carnosol must be formed on one phenolic OH group and one alcoholic OH group. The acetylation of an acid group could take place to form an anhydride, but, under the conditions of the experiment, the anhydride would be decomposed to form its component acids. The fact that there is no alcoholic OH group present has been previously shown. The evidence indicates that the ester shown to be present in the methylated compound is part of the original compound and has not been formed by methylation. The isolation of an unidentified amorphous acidic product which forms a crystalline potassium salt from the saponification of the methylated compound and the indication that a neutral product results from the saponification of carnosol, support this evidence.

The identification of phenanthrene as the product of the sulfur dehydrogenation of the zinc dust distillate indicated that the distillate was a partially saturated phenanthrene. Ultimate analysis of the zinc dust distillate showed that it conformed to the formula $C_{14}H_{18}$. Such a hydrocarbon could be an octahydrophenanthrene. Phenanthrene has been obtained from 1,2,3,4,9,10,11,12-octahydrophenanthrene by dehydrogenation with sulfur by Fulton and Robinson (10). Further evidence that the zinc dust distillate might be an octahydrophenanthrene is contributed by its physical properties. The zinc dust distillate of carnosol boiled

at 298–300° C. Ipatieff (11) reported the boiling point of the octahydrophenanthrene which he obtained by the hydrogenation of phenanthrene to be 290–300° C. Bardhan and Sengupta (12) reported the boiling point of the 1,2,3,4,9,10,11,12-octahydrophenanthrene which they obtained synthetically to be 135° C. at 9 mm. of pressure. They also reported these other properties of their synthetic product: $n_D^{32} = 1.548575$, stable to potassium permanganate, and no decoloration of bromine in chloroform. Cook and Hewett (13) reported the 1,2,3,4,9,10,-11,12-octahydrophenanthrene which they obtained synthetically was an oil which absorbed no bromine, had a boiling point of 159° C. at 15-mm. pressure, and a refractive index of $n_D^{15.2} = 1.5527$. The zinc dust distillate of carnosol gave a refractive index of $n_D^{25} = 1.5584$, absorbed no bromine, and apparently was stable to potassium permanganate. Bergs, *et al.* (14), reported the 1,2,3,4,9,10,11,12-octahydrophenanthrene which they obtained synthetically to be a yellowish oil which showed a brilliant light blue fluorescence in solution. The physical properties of their product conformed to those reported above. The zinc dust distillate of carnosol showed a light blue fluorescence in solution. It also formed no picrate which conforms to the evidence of Schmidt and Mezger (15) observed on the octahydrophenanthrene they obtained by hydrogenation of phenanthrene.

Since no potassium permanganate oxidation product has been obtained to substantiate the above evidence, the presence of an octahydrophenanthrene structure in carnosol cannot be conclusively stated. Further, since the phenanthrene isolated was obtained as a result of degradation reactions which may have caused the formation of phenanthrene by dehydration and ring closure as well as dehydrogenation, the presence of an octahydrophenanthrene nucleus in carnosol can only be said to be indicated. However, based upon the experimental evidence found, (I) is tentatively proposed as the partial structural formula for carnosol.

Of the atoms in the molecular formula $C_{19}H_{26}O_4$, fifteen carbon atoms, eighteen hydrogen atoms and the four oxygen atoms are accounted for. It is possible that the remaining four carbon atoms and eight hydrogen atoms are in the ester side chain. No evidence as to the position of the carboxyl group has been established and no values for $R$ and $R'$ in formula (I) are postulated.



(I)

## SUMMARY AND CONCLUSIONS

The following facts relative to the chemistry of the new compound, carnosol, isolated from *Salvia carnosa* (Dougl.) have been established:

1. The usual physical and chemical properties have been determined.
2. The molecular formula, $C_{19}H_{26}O_4$, has been assigned to this compound as a result of molecular weight determinations and ultimate analyses.
3. Methyl, acetyl and benzoyl derivatives have been made and characterized.
4. It has been established that the compound contains two phenolic OH groups.
5. It has been established also that the compound contains one ester group.
6. Phenanthrene has been obtained from the compound by zinc dust distillation followed by sulfur dehydrogenation.
7. A tentative partial structural formula has been proposed for the new compound.

## REFERENCES

(1) White, A. I., and Jenkins, G. L., JOUR. A. PH. A., 31 (1942), 33.

(2) Clark, E. P., *J. Assoc. Official Agr. Chem.*, 15 (1932), 136.

(3) "Organic Syntheses" (1935), John Wiley and Sons, Inc., New York, N. Y., Vol. XV, p. 3.

(4) Emich, *Monatsch.*, 38 (1917), 219.

(5) Adams, Hunt and Clark, *J. Am. Chem. Soc.*, 62 (1940), 737.

(6) Lyons and Reid, *Ibid.*, 39 (1917), 1727.
(7) Kamm, O., "Qualitative Organic Analysis" (1932), John Wiley and Sons, Inc., New York, N. Y., Second edition, p. 177.
(8) Clar, E., *Ber.*, 72 (1939), 1645.
(9) Kamm, O., "Qualitative Organic Analysis" (1932), John Wiley and Sons, Inc., New York, N. Y., Second edition, p. 195.
(10) Fulton and Robinson, *J. Chem. Soc.* (1933), pp. 1463–1466.
(11) Ipatieff, *et al.*, *Ber.*, 41 (1908), 996.
(12) Bardhan and Sengupta, *J. Chem. Soc.* (1932), p. 2520.
(13) Cook and Hewett, *Ibid.* (1933), p. 1108.
(14) Bergs, *et al.*, *Ber.*, 67 (1934), 238.
(15) Schmidt and Mezger, *Ibid.*, 40 (1907), 4240.

# The Action of Some Organo-Mercurials and Some Copper Salts on Ringworm Fungi*

*By Richard H. Barry,† Walter H. Hartung and Thomas C. Grubb*

Comparative studies have been made relating chemical structure to bactericidal activity, particularly in the phenol series (1, 2). In most cases, investigators have observed a close relationship between bactericidal and fungicidal activity.

Copper and its salts possess bactericidal action (3). The successful use of copper salts as fungicides in the control of plant fungi has prompted the study of these compounds as possible effective agents in the treatment of the dermatoses. Copper deposited on the skin by iontophoresis has been used with apparent success in the treatment of "athlete's foot." Copper sulfate is reported to have given good results in the treatment of skin Aspergilliosis (4). Copper and its salts have, however, proved themselves weak fungicides on the basis of laboratory evaluation against pathogenic fungi (5).

Certain aromatic organic acids, particularly salicylic and benzoic, are employed with favorable results in the treatment of mycoses of the hands and feet. Hence, it appeared logical to investigate the fungicidal properties of a combination of the metal with the acids, that is, copper salicylate and benzoate. The effect of copper salicylate, copper benzoate, salicylic acid and benzoic acid in inhibiting spore suspensions of two organisms commonly associated with fungous diseases of the skin is presented in Table I.

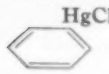
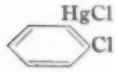
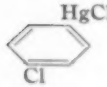
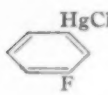
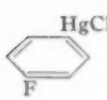
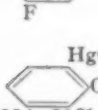
Table I.—Fungicidal-Static Activity of Two Organic Copper Salts

| Compound | Highest Dilutions of Agents Effective in Inhibiting Growth in 10 Minutes | |
|---|---|---|
| | *E. interdigitale* | *A. gypseum* |
| Copper benzoate Suspension 1:20 | 1:40 | .. |
| Copper salicylate Suspension 1:100 | 1:300 | .. |
| Benzoic acid Suspension 1:50 | 1:400 | .. |
| Salicylic acid Suspension 1:50 | 1:700 | .. |
| Copper benzoate 1:50 in dioxane | 1:300 | 1:450 |
| Copper salicylate 1:50 in 50% dioxane | 1:600 | 1:800 |
| Benzoic acid 1:50 in dioxane | 1:500 | 1:700 |
| Salicylic acid 1:100 in 50% dioxane | 1:1500 | 1:2500 |
| Dioxane | No inhibition of growth with 15% dioxane | |

In the field of organic mercurials there are also indications of a parallelism between bactericidal and fungicidal activity, although there are many exceptions. In the present investigation, the primary object was to compare the chlorophenylmercuric chlorides with the corresponding fluorophenyl derivatives and to observe the effect of position isomerism. In Table II are summarized the activities of six aromatic mercurials toward two strains of pathogenic fungi.

* Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.
From the University of Maryland, School of Pharmacy, Baltimore, Md.
† University of Maryland School of Pharmacy Alumni Research Fellow 1940–1941.

Table II.—Fungicidal-Static Activity of Aromatic Mercurials[a]

| Compound 1:1000 Dilution in 75% Acetone | Highest Dilutions (× $10^3$) of Agents Effective in Inhibiting Growth in 10 Minutes *E. interdigitale* | *A. gypseum* |
|---|---|---|
|  | 15 | 20 |
|  | 20 | 25 |
|  | 30 | 35 |
|  | 25 | 25 |
|  | 35 | 40 |
|  1:1000 in 20% acetone | 20 | 25 |
| $HgCl_2$ 1:1000 in water | 15 | 15 |
| Acetone | No inhibition of growth with 15% acetone | |

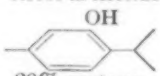
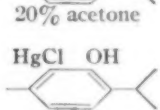
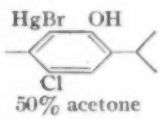
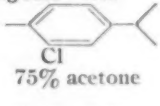
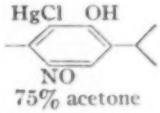
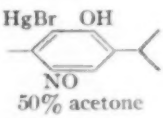
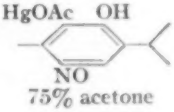
[a] Prepared by Dr. M. F. W. Dunker.

In Table III is summarized the fungicidal activity of three mercurated chlorothymols and nitrosothymols. The effect of replacement of halogen with nitroso group on the activity is shown.

Copper benzoate and copper salicylate were prepared by the reaction of copper sulfate with sodium benzoate and sodium salicylate, respectively. Copper benzoate, ($[C_6H_5COO]_2Cu.2H_2O$), is a blue powder, insoluble in water and alcohol, slightly soluble in acetone, soluble in dioxane. Copper salicylate, ($[C_6H_4OHCOO]_2Cu.4H_2O$), is slightly soluble in water and alcohol, soluble in dioxane. The mercurials tested are very slightly soluble in water, soluble in acetone.

The near insolubility of many organic metallic compounds in water necessitates the testing of stock dilutions of these compounds either as suspensions in water or oils or in solution in some organic solvent. Suspensions of the mercurials listed in Table II were employed by Dunker and Grubb (6) in testing antibacterial activity. It was found in the present investigation that acetone and dioxane, which were employed to prepare the stock dilutions of the compounds, did not exert fungicidal activity in concentrations below 15% in the time interval used (10 minutes). Dioxane has previously been used by investigators at Purdue as a solvent in testing bactericidal activity (7).

Table III.—Fungicidal-Static Activity of Mercurated Thymols[a]

| Compound 1:1000 in Acetone | Highest Dilutions (× $10^3$) of Agents Effective in Inhibiting Growth in 10 Minutes *E. interdigitale* | *A. gypseum* |
|---|---|---|
|  20% acetone | 3 | 4 |
|  50% acetone | 35 | 40 |
|  50% acetone | 30 | 45 |
|  75% acetone | 25 | 30 |
|  75% acetone | 30 | 30 |
|  50% acetone | 20 | 25 |
|  75% acetone | 15 | 15 |
| Acetone | No inhibition of growth with 15% acetone | |

[a] Prepared by Dr. A. W. Ruddy.

## EXPERIMENTAL

*Preparation of Stock Dilutions.*—Suspensions of the copper salts were made in water. Five-tenths gram of tragacanth per 100 cc. of water was found sufficient to make a stable suspension of the copper

compounds. Stock solutions of the copper salts in dioxane and of the mercurials in acetone were made as indicated in the tables.

*Determination of Activity.*—The organisms used were *Epidermophyton interdigitale* and *Achorion gypseum*. These organisms are commonly associated with "athlete's foot." (We are indebted to Dr. Adelia McCrea and to Dr. George Reddish for these cultures.) The method of testing employed was essentially that of McCrea (8). The organisms were grown on Sabouraud's dextrose agar in Kolle flasks. After two weeks' incubation at room temperature (22–27° C.), spore suspensions were prepared. One-half cc. of spore suspension was inoculated into 2 cc. of test dilution and gently mixed by tapping the tube. At intervals of ten minutes, one loopful of the mixture was removed and subcultured on Sabouraud's agar slants, which were then incubated in the dark at room temperature for 10 to 14 days. The results are shown in the accompanying tables.

*Discussion of Results.*—The dilutions shown in the tables are to be considered as fungicidal-static, a term which is intended to mean that the compounds tested in the manner described, exert fungistatic as well as fungicidal activity. It is obvious that some of the compound tested is carried over to the agar slants when subcultures are made, thereby enabling it to exert fungistatic acitivity.

The difference in response to the copper salts of the two fungi tested is not significant. However, it can be seen that *E. interdigitale* was the more resistant. There is noted a difference in activity of the copper salts according to the manner in which the stock preparations were made. Suspensions of the salts were found not as active as were the same compounds dissolved in dioxane. Salicylic acid was found superior to benzoic acid as a fungicide, both in suspension and in solution. Similarly, copper salicylate is more fungicidal than copper benzoate. But both copper salts are inferior to their corresponding acids as fungicides *in vitro*.

The introduction of halogen or hydroxyl on the ring in phenylmercuric halides increases the fungicidal-static activity. The *p*-chloro and *p*-fluoro substituted compounds were found more active than the *o*-chloro and *m*-fluoro substituted isomers, respectively. *p*-Fluorophenyl mercuric chloride was the most active of the series tested. These results parallel the anti-bacterial activity observed with these compounds (6). The fungicidal activity observed with *p*-hydroxyphenylmercuric chloride and mercuric chloride agrees with earlier reports on these compounds (9, 10).

Of the mercurated thymols tested, chlorothymolmercuric chloride was found to be superior to the bromide or acetate. Substitution of a nitroso group for Cl lowers the activity of the thymol. In this respect the nitroso group (NO) is probably similar to the nitro group ($NO_2$) (2, 11). Chlorothymolmercuric chloride was found to be the most active of all the mercurials tested. *E. interdigitale* was more resistant to the mercurials than *A. gypseum*.

## CONCLUSIONS

1. Copper salicylate and copper benzoate were prepared and a study of the fungicidal-static activity against two dermatophytes shows them to be inferior to salicylic and benzoic acids as fungicides.

2. Introduction of halogen or hydroxyl into phenylmercuric chloride increases fungicidal-static activity. Halogen substitution increases the activity in the order ortho, meta, para. *p*-Fluorophenylmercuric chloride was found more active than *p*-chlorophenylmercuric chloride. Anti-bacterial and fungicidal activity were observed to be parallel in the case of the *p*-fluoro and *p*-chloro mercuric halides.

3. The mercurated chlorothymols are good fungicides. The order of increasing activity in this series is acetate, bromide, chloride. Substitution of a nitroso group for halogen lowers the activity of the thymol.

## REFERENCES

(1) Klarmann, E., *et al.*, *J. Lab. Clin. Med.*, 20 (1934), 40.

(2) Woodward, G. J., *et al.*, *Ibid.*, 19 (1934), 1216.

(3) DeWitt, L. M., and Sherman, H. J., *J. Infect. Diseases*, 18 (1916), 369.

(4) Meyers, J. T., and Dunn, A. D., *J. Am. Med. Assoc.*, 95 (1930), 794.

(5) Emmons, C. W., *Arch. Dermatol. and Syphilol.*, 28 (1933), 15.

(6) Dunker, M. F. W., and Grubb, T. C., *J. Bact.*, 39 (1940), 243.

(7) Feasley, C. F., *et al.*, JOUR. A. PH. A., 30 (1941), 41.

(8) McCrea, Adelia, *J. Lab. Clin. Med.*, 17 (1931), 72.

(9) Dunn, C. G., *J. Infect. Diseases*, 61 (1937), 31.

(10) Schamberg, J. F., and Kolmer, J. A., *Arch. Dermatol. and Syphilol.*, 6 (1922), 746.

(11) Koehler, H. F., and Hartung, W. H., *J. Am. Chem. Soc.*, 63 (1941), 872.

# The Effect of Estrone and Stilbestrol on the Response of Rabbits' Uteri to Ergonovine

*By Harry J. Wick and Clarence E. Powell*†*

It is a common experience in the assaying of oxytocic drugs on isolated uteri of guinea pigs and rabbits that a considerable number of the organs have to be discarded because of their erratic responses. For example, if the animal is immature, the uterus invariably requires large doses for a contraction, and after two or three trials it fails to respond any further. On the other hand, a mature animal gives rise to a uterus that may be highly congested and exercises vigorous, spontaneous contractions. In both instances, the organ is not suitable for a quantitative estimation of active substances. Only by prolonged segregation of young females from males, and by further selection of the uterus upon sacrifice, can one succeed in making a satisfactory test. A relatively pale but fully developed pair of uterine horns, as a rule, is most preferable. The method is wasteful and costly.

Reynolds (1) has summarized the present knowledge on the mammalian uterus, particularly the rabbit's. He repeatedly emphasizes the fact that the uterus becomes motile and irritable when it is under the influence of estrogenic substances, and that its responsiveness to posterior pituitary extracts is high if it has high irritability. It was based upon this principle that the present series of experiments was undertaken. The proposal of assaying ergonovine, a new ergot alkaloid, on the isolated rabbit's uterus was made by Swanson, Hargreaves and Chen (2). It occurred to us that if immature female rabbits were treated with estrone or stilbestrol, their uteri might be relatively more irritable and thus more responsive to ergonovine, just as they would be to pituitary extracts.

## EXPERIMENTAL

To test this point, 159 New Zealand red rabbits, 6 of which were ovariectomized, were injected subcutaneously with various doses of estrone. Another group of 25 rabbits was similarly treated with stilbestrol. A third group of 10 animals was used as the control. In order to rule out any difference of reaction due to the strain of the animal, 20 albino rabbits were given estrone by subcutaneous injection. All animals weighed between 1.5 and 1.8 Kg. Both estrone and stilbestrol were dissolved in cottonseed oil, the concentration being 1:100,000 each. The total dose of either drug as listed in Table I was divided into six fractions, and one fraction was injected every second day. Two days after the last injection, namely, on the thirteenth day, the rabbit was scarified, and its uterus removed. A segment of the ovarian end was immersed in 100 cc. of Tyrode's solution kept at 37.5° C., and arranged for recording in precisely the same manner as the U. S. P. directs for the assay of posterior pituitary solution (3).

Table I.—Ergonovine Assay on Isolated Uteri of Rabbits Treated with Estrone and Stilbestrol

| Number of Rabbits Used | Pre-Assay Medication: Drug | Pre-Assay Medication: Total Dose, γ per Animal | Uteri Suitable for Assay: Number | Uteri Suitable for Assay: Per Cent |
|---|---|---|---|---|
| 10 (Control) | None | .. | 0 | 0 |
| 10 | Estrone | 3 | 0 | 0 |
| 10 | | 6 | 0 | 0 |
| 20 | | 9 | 15 | 75 |
| 45 | | 12 | 42 | 93.3 |
| 28 | | 15 | 25 | 89.3 |
| 10 | | 18 | 6 | 60 |
| 10 | | 24 | 5 | 50 |
| 10 | | 30 | 6 | 60 |
| 10 | | 60 | 4 | 40 |
| 5[a] | Estrone | 6 | 0 | 0 |
| 5[a] | | 9 | 3 | 60 |
| 5[a] | | 12 | 4 | 80 |
| 5[a] | | 15 | 4 | 80 |
| 6[b] | Estrone | 12 | 5 | 83.3 |
| 5 | Stilbestrol | 3 | 0 | 0 |
| 5 | | 6 | 2 | 40 |
| 5 | | 9 | 5 | 100 |
| 5 | | 12 | 4 | 80 |
| 5 | | 15 | 3 | 60 |

[a] White color.
[b] Ovariectomized.

After the muscle was completely relaxed, a dose of 1 cc. of 1:10,000 solution of ergonovine maleate was applied. Following a wash-out and a rest of 15 minutes, the dose was repeated. If the response matched that of the first dose, smaller amounts,

* The authors are indebted to Dr. K. K. Chen for his interest and substantial assistance in the preparation of this manuscript.

† From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.

equivalent to 4/5, 3/5 or less, were then tried. The muscle would be considered satisfactory provided the heights of contractions showed a quantitative relationship with the doses employed. An example is well illustrated in Fig. 1. It must be emphasized that the height of contraction (peak) is the only criterion, and that the same dose of ergonovine makes the muscle repeat its response with exactness.

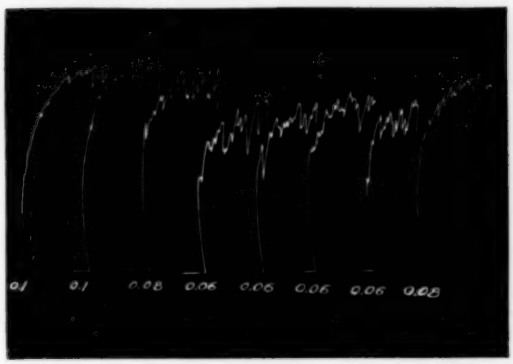



Fig. 1.—An Example of a Utilizable Strip of the Isolated Rabbit's Uterus.

The numbers indicate doses of ergonovine maleate in milligrams. Note the quantitative relationship with the amounts of the drug applied.

## DISCUSSION

As shown in Table I, the results conclusively prove that subcutaneous injection of estrone or stilbestrol renders the immature rabbit's uterus more irritable, more reliable in response, and thus more utilizable in the assay of ergonovine. The optimal dose of estrone per animal is obviously in the neighborhood of 12γ, and that of stilbestrol, 9γ, each divided into 6 portions and injected in 11 days. Ovariectomy did not nullify the estrone action for 83% of the uteri was fully reactive. Although the white rabbits appeared slightly less benefited, the number of satisfactory preparations reached 80%. This is in strong contrast with the 10 control rabbits, none of which had a uterus suitable for assaying purposes. The economic advantage of the estrogenic therapy in rabbits cannot therefore, be overestimated. It is also probable that the ergonovine assay is attended by greater precision.

## SUMMARY

1. Immature rabbits injected subcutaneously with divided doses of estrone or stilbestrol give rise to an overwhelmingly high percentage of uteri, which, when isolated, are suitable for ergonovine assays.

2. The optimal dose (total) of estrone is approximately 12γ per animal, and that of stilbestrol, 9γ.

3. The chief advantage of this procedure is economy. In addition, the assay may possibly be carried out with higher precision.

## REFERENCES

(1) Reynolds, S. R. M., "Physiology of the Uterus" (1939), Paul B. Hoeber, Inc., New York, N. Y., pp. 316, 319 and 320.

(2) Swanson, E. E., Hargreaves, C. C., and Chen, K. K., JOUR. A. PH. A., 24 (1935), 835.

(3) "United States Pharmacopœia XI" (1936), p. 217.

# Assay Methods for Phenothiazine Pharmaceuticals*

*By Lewis E. Harris† and Eloise B. Kerl‡*

Phenothiazine has recently assumed an important role in the veterinary pharmaceutical field since it has been found to be a very efficient anthelmintic with a comparatively low toxicity (1). To date there are no published assay methods for determination of phenothiazine purity or of phenothiazine content in pharmaceuticals. Determinations of sulfur or nitrogen by standard methods were found to be unsatisfactory and, so far, colorimetric methods have been of no value. This report describes a method for the routine control assay of phenothiazine in pharmaceutical preparations, based on the extraction and subsequent weighing of the drug. Two principal types of pheno-

* Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.

† Director of Research and Control Department, Norden Laboratories, Lincoln, Neb.

‡ Control Chemist, Norden Laboratories, Lincoln, Neb.

thiazine preparations are marketed—one a tablet or bolus form, the other an aqueous suspension. The following assay procedures may be used for both types of preparations.

## EXPERIMENTAL

(*a*) *Tablets, Boluses or Granulations.*—Weigh and reduce to a fine powder not less than 20 tablets, 5 boluses or 10 Gm. of granulation. Weigh a sample of this powder equivalent to about 1.5 Gm. phenothiazine and transfer to fat-free filter paper. Fold the paper carefully to enclose sample and place in extraction thimble. Carry out the extraction by means of an A. S. T. M. extraction apparatus using anhydrous acetone as the solvent. The extraction should be continued about eight hours.

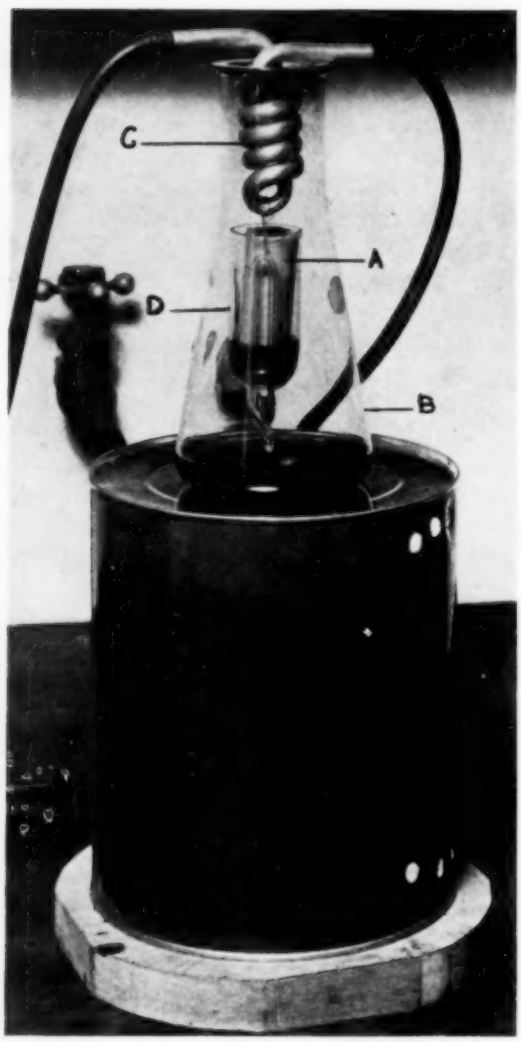



Fig. 1.—The A. S. T. M. Extraction Apparatus.

The dried material to be extracted is placed in the thimble (*a*). The acetone in the tared flask (*b*) is vaporized by low heat, condensed on the coil (*c*) and drops into the glass siphon cup (*d*).

For accuracy, the extraction flask used with the above apparatus should not weigh more than 75 Gm. After extraction is complete, the acetone is evaporated, the residue dried to constant weight and the percentage of phenothiazine calculated. In drying to constant weight, the flask should be placed in a horizontal position and the temperature kept below 80° C. to prevent loss by sublimation.

(*b*) *Suspensions.*—Agitate the sample to insure complete mixing and measure or weigh at least a 10-cc. sample. Transfer the sample to an ordinary filtering funnel fitted with a filtering cone and two sheets of fat-free filter paper of moderate retention grade (Whatman's No. 2 is satisfactory). Conduct the filtration with moderate suction and wash the residue with distilled water until all water-soluble material has been removed. Remove paper and residue, fold into a shape which will ultimately fit into the extraction thimble, place in an evaporating dish and dry at temperature under 80° C. Finally place the dried paper and residue in the extraction thimble and proceed with the extraction as directed in (*a*).

## RESULTS

The results of over two hundred individual assays show this method to be satisfactory for routine control work. The tables show typical results of assays on phenothiazine suspensions, granulations, tablets and boluses.

Table I.—Phenothiazine Suspension

| Sample No. | Phenothiazine Content (Grams per Fluid Ounce) Theory | Assay | Per Cent Recovery |
|---|---|---|---|
| 9413 | 12.00 | 12.11 | 100.8 |
| 9419 | 12.00 | 11.91 | 99.2 |
| 9469 | 12.00 | 11.81 | 98.4 |
| 9968 | 12.00 | 11.99 | 99.9 |
| 9575 | 12.00 | 12.13 | 101.0 |
| 9578 | 12.00 | 12.03 | 100.2 |

Table II.—Phenothiazine Granulation

| Sample No. | Phenothiazine Content Theory | Assay | Per Cent Recovery |
|---|---|---|---|
| 265 L | 70.50 | 70.51 | 100.0 |
| 275 L | 70.50 | 70.25 | 99.6 |
| 277 L | 70.50 | 70.96 | 100.6 |
| 280 L | 70.50 | 70.71 | 100.3 |
| 289 L | 70.50 | 70.68 | 100.2 |
| 328 L | 70.50 | 70.94 | 100.6 |

Table III.—Phenothiazine Tablets

| Sample No. | Phenothiazine Content (Grains per Tablet) Theory | Assay | Per Cent Recovery |
|---|---|---|---|
| 9240 | 0.500 | 0.505 | 101.0 |
| 9241 | 0.500 | 0.503 | 100.6 |
| 9282 | 0.500 | 0.505 | 101.0 |
| 9355 | 0.500 | 0.497 | 99.4 |
| 9364 | 0.500 | 0.510 | 102.0 |
| 9447 | 0.500 | 0.504 | 100.8 |

Table IV.—Phenothiazine Boluses

| Sample No. | Phenothiazine Content (Grams per Bolus) Theory | Assay | Per Cent Recovery |
|---|---|---|---|
| 8852 | 12.00 | 12.02 | 100.1 |
| 9239 | 12.00 | 12.23 | 101.9 |
| 9288 | 12.00 | 12.16 | 101.3 |
| 9528 | 12.00 | 12.20 | 101.6 |
| 9911 | 12.00 | 12.22 | 101.8 |
| 9213 | 12.00 | 12.07 | 100.6 |

Each sample was run in duplicate. Results of duplicate assays show a maximum variation of ± 2.0%.

### DISCUSSION AND SUMMARY

This procedure for the assay of phenothiazine, based on the extraction and subsequent weighing of the drug, was found to give good recoveries of phenothiazine from suspensions, granulations, tablets and boluses. The method, of course, would not be satisfactory if acetone-soluble substances other than phenothiazine were present in the preparation. This is not usually the case, hence this method should be useful in the majority of preparations.

### REFERENCE

(1) Harwood, "Phenothiazine as an Anthelmintic," *Bulletin of U. S. D. A., B. A. I.* (September 1940).

# The Utilization of Pigeons for the Biological Assay of *Adonis Vernalis*, N. F. VI*

*By W. M. Benson† and L. D. Edwards‡*

Since *Adonis vernalis*, N. F. VI, is a potent cardiac drug, it is contended that an assay method should be adopted for its standardization. Inasmuch as the potency of the drug cannot be determined to an accurate degree by chemical analysis, several biological procedures have been suggested. The following work deals with the application of the pigeon emetic method together with minor comparisons with the one-hour frog and cat methods in the assay of *Adonis*.

### EXPERIMENTAL

Each of the three tinctures was assayed by the pigeon emetic and one-hour frog and cat methods.

*The Pigeon Emetic Method.*—The procedure introduced by Hanzlik (1) in 1929 was the method used in this work, although certain modifications were made. Following the suggestion of Lieb and Mulinos (2) the assay birds were previously standardized to a maximum and minimum dose of ouabain, thereby allowing the rejection of all hypo- and hypersensitive birds. The two standardization doses selected were 0.065 mg. per Kg. and 0.030 mg. per Kg. Those birds that did not emese to the larger dose and those birds which did emese to the smaller dose were rejected from use.

The "Minimum Emetic Dose" selected represents the smallest amount of *Adonis*, expressed in cc. of the tincture per Kg. of bird body weight which is capable of producing emesis in 75% of a group of eight birds within a period of 15 minutes following the injection of the drug. The results obtained are as follows:

Table I.—The Determination of the Minimum Emetic Dose of Tincture of Adonis

| Dose of Tincture, Cc./Kg. | Number of Injections | Results Emesis | Results No Emesis |
|---|---|---|---|
| Tincture A | | | |
| 0.210 | 8 | 2 | 6 |
| 0.215 | 8 | 7 | 1 |
| 0.220 | 8 | 4 | 4 |
| 0.225 | 8 | 5 | 3 |
| 0.230 | 8 | 4 | 4 |
| 0.235 | 8 | 4 | 4 |
| 0.240 (M. E. D.) | 8 | 7 | 1 |
| 0.245 | 8 | 6 | 2 |
| 0.250 | 8 | 5 | 3 |
| 0.255 | 8 | 7 | 1 |
| 0.260 | 8 | 8 | 0 |
| Tincture B | | | |
| 0.165 | 8 | 2 | 6 |
| 0.170 | 8 | 5 | 3 |
| 0.175 | 8 | 4 | 4 |
| 0.180 | 8 | 3 | 5 |
| 0.185 | 8 | 1 | 7 |
| 0.190 | 8 | 4 | 4 |
| 0.195 | 8 | 4 | 4 |
| 0.200 | 8 | 5 | 3 |
| 0.205 (M. E. D.) | 8 | 6 | 2 |
| 0.210 | 8 | 7 | 1 |

* Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.

† An abstract of a thesis submitted to the Graduate Council of the University of Florida in partial fulfilment of the requirements for the degree of Master of Science.

‡ Professor of Pharmacognosy and Pharmacology, University of Florida.

Table I.—(*Continued*)

| Dose of Tincture, Cc./Kg. | Number of Injections | Results Emesis | Results No Emesis |
|---|---|---|---|
| Tincture C | | | |
| 0.140 | 8 | 1 | 7 |
| 0.145 | 8 | 1 | 7 |
| 0.150 | 8 | 4 | 4 |
| 0.155 | 8 | 5 | 3 |
| 0.160 | 8 | 3 | 5 |
| 0.165 (M. E. D.) | 8 | 6 | 2 |
| 0.170 | 8 | 6 | 2 |
| 0.175 | 8 | 5 | 3 |
| 0.180 | 8 | 6 | 2 |
| 0.185 | 8 | 7 | 1 |
| 0.190 | 8 | 7 | 1 |

*The One-Hour Frog Method.*—The frog procedure employed followed closely the official U. S. P. XI method for the assay of digitalis except that the frogs were standardized to ouabain, having a M. S. D. of 0.0005 mg. per Gm. of frog.

The "Minimum Systolic Dose" as herein used is defined as the smallest quantity of drug, expressed in cc. of tincture per Gm. of frog weight which is capable of producing systolic stoppage of the ventricle in 75% of a group of four frogs, at exactly one hour following the injection. The results obtained are given in Table II.

Table II.—The Determination of the Minimum Systolic Dose of Tincture of Adonis

| Cc. of Tincture per Gm. of Frog | Number of Injections | Results S. S. | Results Beats |
|---|---|---|---|
| Tincture A | | | |
| 0.0020 | 4 | 0 | 4 |
| 0.0025 | 4 | 1 | 3 |
| 0.0030 (M. S. D.) | 4 | 4 | 0 |
| 0.0035 | 4 | 4 | 0 |
| Tincture B | | | |
| 0.0015 | 4 | 0 | 4 |
| 0.0020 | 4 | 1 | 3 |
| 0.0025 | 4 | 2 | 2 |
| 0.0030 (M. S. D.) | 4 | 3 | 1 |
| 0.0035 | 4 | 4 | 0 |
| Tincture C | | | |
| 0.0015 | 4 | 1 | 3 |
| 0.0020 | 4 | 0 | 4 |
| 0.0025 (M. S. D.) | 4 | 3 | 1 |
| 0.0030 | 4 | 3 | 1 |
| 0.0035 | 4 | 4 | 0 |

*The Cat Method.*—The method used was much like that originally described by McGuigan and McGuigan (3) using cats instead of dogs. In this method of assay the anesthetic used was sodium pentobarbital, 35 mg. per Kg. Artificial respiration was maintained throughout the assay period. The tinctures were administered by femoral injection at the rate of 0.1 cc. per Kg. of cat weight every five minutes until death. The average fatal doses for the three tinctures are given in Table III.

Table III.—The Determination of the Minimum Fatal Dose of Tincture of Adonis

| Weight of Cat, Kg. | Number of Injections, 0.1 Cc./Kg. | Fatal Dose, Cc. Tr./Kg. | Average Fatal Dose, Cc. Tr./Kg. |
|---|---|---|---|
| Tincture A | | | |
| 1.92 | 11 | 1.1 | |
| 2.10 | 14 | 1.4 | 1.20 |
| 2.18 | 11 | 1.1 | |
| Tincture B | | | |
| 2.95 | 11 | 1.1 | |
| 2.55 | 10 | 1.0 | 1.10 |
| 2.20 | 12 | 1.2 | |
| Tincture C | | | |
| 3.90 | 11 | 1.1 | |
| 2.35 | 11 | 1.1 | 1.10 |
| 1.55 | 11 | 1.1 | |

*Discussion of Results.*—Arbitrarily selecting Tincture of Adonis (B) as 100% in strength, the per cent deviation in potency of the three tinctures has been determined when assayed by the three different methods as outlined (see Table IV).

Table IV.—Estimation of the Per Cent Potency and Deviation of Tinctures of Adonis (A), (B) and (C) When Assayed by Three Different Methods. Tincture B is Arbitrarily Selected as Being 100% in Strength

| | Tincture | Cc. Tr./Kg. | Per Cent Potency | Per Cent Deviation |
|---|---|---|---|---|
| Frog method, M. S. D. | A | 3.00 | 100.0 | 0.0 |
| | B | 3.00 | 100.0 | 0.0 |
| | C | 2.50 | 120.0 | 20.0 |
| Cat method, M. F. D. | A | 1.20 | 91.6 | 8.4 |
| | B | 1.10 | 100.0 | 0.0 |
| | C | 1.10 | 100.0 | 0.0 |
| Pigeon method, M. E. D. | A | 0.225 | 85.3 | 14.7 |
| | B | 0.190 | 100.0 | 0.0 |
| | C | 0.165 | 115.0 | 15.0 |

Theoretically, the per cent deviation from the standard should be the same for the several assay methods. In the comparison drawn here, the per cent potency of Tincture of Adonis (A) assays 100% by the frog method, 91.6% by the cat method, and 85.3% by the pigeon emetic method. Tincture C assays 120% by the frog method, 100% by the cat method and 115% by the pigeon method. Although the potency varies somewhat with the method of assay the percentage strengths run fairly parallel. In no case has the extrene per cent of deviation been greater than 20%.

In the pigeon method when birds were given only short rest periods between injections a conditioning effect was observed.

These results, however, indicate that the pigeon emetic method is suitable for the assay of *Adonis vernalis*.

## CONCLUSIONS

1. The results of the assay of tinctures of *Adonis* by the pigeon emetic method parallel the results obtained by the one-hour frog and fatal cat methods.

2. Because the pigeon method possesses a definite end-point, is simple, economical and reasonably accurate, its use for the assay of *Adonis vernalis* appears worthy of consideration for adoption in the National Formulary as the official method.

3. Inasmuch as the standardization of the birds to a maximum and minimum dose of ouabain seems important, the recommendation is made that this procedure be carried out prior to the assay of the drug. It is further suggested that a rest period of one month be allowed between successive injections.

## REFERENCES

(1) Hanzlik, P. J., *J. Pharmacol.*, 35 (1929), 363.

(2) Lieb, C. C., and Mulinos, M. G., *Ibid.*, 48 (1933), 282.

(3) McGuigan, R. A., and McGuigan, H. A., *Ibid.*, 63 (1938), 76.

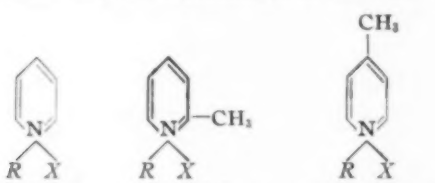
---

# Germicidal Activity of Some Quaternary Ammonium Salts*

*By H. G. Kolloff, A. P. Wyss, R. E. Himelick and F. Mantele*

Following the report of the germicidal activity of quaternary ammonium salts by Domagk in 1935 (1), considerable attention has been directed toward compounds of this type.

Since the introduction of a methyl group into the phenol molecule increases the germicidal activity (2, 3), it was thought desirable to compare the activity of certain alkyl pyridinium halides with the corresponding alpha- and gamma-picolinium compounds. The chemical relationship of these quaternary salts may be represented as follows:




Pyridinium alkyl halides | α-Picolinium alkyl halides | γ-Picolinium alkyl halides

in which $R$ = $C_{12}H_{25}$-, $C_{14}H_{29}$- or $C_{16}H_{33}$-, and X = halogen.

The method used in the preparation of the 27 compounds herein reported was essentially that reported by Knight and Shaw (4).

Preliminary germicidal tests were made according to the F. D. A. method using *Staphylococcus aureus* at 37° C. (5). Each test was accompanied by a special control to be certain that the dilution reported was germicidal. The preliminary results indicate that the introduction of a methyl group into the aromatic nucleus of these alkyl pyridinium halides does not in general increase the germicidal activity.

* From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

## EXPERIMENTAL

*Materials:*

*Pyridine*—Mallinckrodt, analytical reagent.

*α-Picoline*—Eastman Kodak Co., redistilled: b. p., 126–127.5° C.; $n_D^{20}$, 1.5009.

*γ-Picoline*—Pure γ-picoline was prepared by the procedure recently described by Kolloff and Hunter (6); b. p., 142° C.; $n_D^{20}$, 1.5052.

*Tetradecyl chloride* (b. p. 126–129° C. at 1.5 mm.; $n_D^{20}$, 1.4465) and

*Hexadecyl chloride* (b. p. 120–124° C. at 0.07 mm.; $n_D^{20}$, 1.4501) were prepared by the method of Norris and Taylor (7).

*Dodecyl iodide* (b. p. 142–144° C. at 5.0 mm.; yield, 32.6 Gm.) and

*Tetradecyl iodide* (b. p. 178–181° C. at 13.0 mm.; yield, 31.5 Gm.) were obtained from 25.0 Gm. quantities of the corresponding alcohols using the method of Levene and West (8).

*All other alkyl halides* were obtained from the Eastman Kodak Company and were used as supplied without further purification.

*Preparation of the Quaternary Ammonium Salts.*—The procedure was similar to that used by Knight and Shaw (4), and the following description for the preparation of dodecyl α-picolinium iodide serves as an illustration:

*Dodecyl α-Picolinium Iodide.*—Three grams of dodecyl iodide and 1.0 Gm. of α-picoline were mixed

in a flask equipped with an air condenser and heated on a steam bath over night. The solid product was treated with several portions of cold absolute ether to remove unreacted materials, filtered and air dried. Yield of crude product, 3.3 Gm.; m. p., 122–126° C. (corr.). The product was purified to constant melting point by repeated solution in absolute alcohol, treatment with charcoal and precipitation with absolute ether. Yield of pure product, 2.7 Gm.; m. p. 130–131° C. (corr.). Analysis: calculated for $C_{18}H_{32}IN$, 33.83% iodine; found, 33.90%.

As pointed out by Knight and Shaw (4), the alkyl chlorides required a temperature of 150° C. for at least eight hours. Even after this treatment, the yields were comparatively low. In general, the alkyl iodides gave the best yields and the bromides next best.

*Preliminary Tests for Germicidal Action.*—In general, 1:1000 stock solutions of the salts were prepared in water or, in cases of too low solubility, 50% alcohol. Suitable aqueous dilutions of the stock solution were subjected to the F. D. A method for testing germicidal activity (5) using *Staphylococcus aureus* at 37° C. Phenol controls were made with each determination as a check on the viability of the test organism. The approximate dilutions of the quaternary ammonium salts which were capable of killing the test organism in ten minutes, but not in five minutes, are reported in Table IV. In order to be certain that the dilution reported was germicidal, the following control was performed:

One-half cc. of the suspension of *Staphylococcus aureus* test organism was thoroughly mixed with 5 cc. of distilled water. One standard loopful of this mixture was transferred to a tube of 10 cc. of sterile nutrient broth, to which was added one standard loopful of the dilution of the compound being tested. This mixture was incubated for 48 hours after which positive or negative growth indicated the absence or presence of bacteriostatic activity under the conditions of the experiment.

The results of such tests performed with the compounds described in this paper show that each of the dilutions reported in Table IV is definitely germicidal.

Table I—Pyridinium Alkyl Halides

| Pyridine plus: | Melting Points, ° C. (Corr.) First | Second | Per Cent Halogen Calcd. | Found |
|---|---|---|---|---|
| $C_{12}H_{25}$-Cl | 78–80[a] | 147–148 | 12.50 | 12.51 |
| $C_{12}H_{25}$-Br | 44.5–45.5[b] | 125.5 | 24.35 | 24.08 |
| $C_{12}H_{25}$-I | 88–89[c] | 93 | 33.83 | 33.90 |
| $C_{14}H_{29}$-Cl | 77–78[d] | 200–201 | 11.38 | 11.41 |
| $C_{14}H_{29}$-Br | 54.5–55.5 | 198 | 22.44 | 22.16 |
| $C_{14}H_{29}$-I | 95.5–96[e] | 161 | 31.48 | 31.31 |
| $C_{16}H_{33}$-Cl | 82–83[f, h] | 217 | 10.44 | 10.08 |
| $C_{16}H_{33}$-Br | 60.5–61.5 | 217 | 20.80 | 20.60 |
| $C_{16}H_{33}$-I | 89–90[g] | 211 | 29.43 | 29.30 |

Knight and Shaw (4) reported: [a] (71°, 145°); [b] (89–90°, 125°); [c] (88–89°, 93°); [d] (77°, 205°); [e] (94°, 155°); [f] (83°, 217°); [g] (98°, 205°).

[h] French patent (9); no constants given.

Table II—α-Picolinium Alkyl Halides

| α-Picoline plus: | Melting Points, ° C. (Corr.) First | Second | Per Cent Halogen Calcd. | Found |
|---|---|---|---|---|
| $C_{12}H_{25}$-Cl | 94.5–95.5[a, b] | 141 | 11.91 | 11.67 |
| $C_{12}H_{25}$-Br | 123–124 | ... | 23.35 | 23.62 |
| $C_{12}H_{25}$-I | 130–131 | ... | 32.61 | 32.30 |
| $C_{14}H_{29}$-Cl | ...[c] | ... | 10.89 | 10.11[d] |
| $C_{14}H_{29}$-Br | 125–126 | 192.5 | 21.59 | 21.29 |
| $C_{14}H_{29}$-I | 130–131 | 146.5 | 30.42 | 30.60 |
| $C_{16}H_{33}$-Cl | ...[c] | ... | 10.02 | 9.80 |
| $C_{16}H_{33}$-Br | 123.5–124.5 | 214 | 20.07 | 19.92 |
| $C_{16}H_{33}$-I | 119–120 | 201 | 28.51 | 28.33 |

[a] French patent (9); no constants given.

[b] Hygroscopic; dried in vacuum desiccator, filled m. p. tube, again dried in vacuum dessicator, sealed in flame, and determined m. p. as usual.

[c] Extremely hygroscopic; liquefied in air so rapidly that m. p. tube could not be charged to follow the procedure described under "b."

[d] Low yield and, with the very hygroscopic nature, this was the best analysis obtained.

Table III—γ-Picolinium Alkyl Halides

| γ-Picoline plus: | Melting Points, ° C. (Corr.) First | Second | Per Cent Halogen Calcd. | Found |
|---|---|---|---|---|
| $C_{12}H_{25}$-Cl | 61–63[a] | ... | 11.91 | 11.75 |
| $C_{12}H_{25}$-Br | 66–67 | ... | 23.35 | 23.08 |
| $C_{12}H_{25}$-I | 61.5–62 | ... | 32.61 | 32.38 |
| $C_{14}H_{29}$-Cl | 73–74[a] | ... | 10.89 | 11.06 |
| $C_{14}H_{29}$-Br | 79–80 | ... | 21.59 | 21.51 |
| $C_{14}H_{29}$-I | 74–75 | ... | 30.42 | 30.30 |
| $C_{16}H_{33}$-Cl | 81–82 | 104–105 | 10.02 | 9.82 |
| $C_{16}H_{33}$-Br | 84.5–85.5 | 110 | 20.07 | 20.07 |
| $C_{16}H_{33}$-I | 67–68 | 110–110.5 | 28.51 | 28.41 |

[a] Hygroscopic; dried in vacuum desiccator, filled m. p. tube, again dried in vacuum desiccator, sealed in flame, and determined m. p. as usual.

Table IV—Approximate Germicidal Dilutions

| Alkyl Halides | Pyridine | α-Picoline | γ-Picoline |
|---|---|---|---|
| $C_{12}H_{25}$-Cl | 20,000 | 25,000 | 20,000 |
| $C_{12}H_{25}$-Br | 20,000 | 15,000 | 15,000 |
| $C_{12}H_{25}$-I | 10,000 | 10,000[a] | 15,000 |
| $C_{14}H_{29}$-Cl | 50,000 | 45,000[b] | 50,000 |
| $C_{14}H_{29}$-Br | 45,000 | 50,000 | 40,000 |
| $C_{14}H_{29}$-I | 50,000[a] | 40,000[a] | 40,000[a] |
| $C_{16}H_{33}$-Cl | 50,000 | 50,000 | 50,000 |
| $C_{16}H_{33}$-Br | 50,000[a] | 50,000 | 50,000[a] |
| $C_{16}H_{33}$-I | 20,000[a] | 20,000[a] | 20,000[a] |

[a] Used 50% alcohol as solvent for stock solution.

[b] Low halogen analysis, *cf.* Table II.

## SUMMARY

A group of 27 alkyl pyridinium- and alkyl picolinium-halides were synthesized and preliminary germicidal tests made to discover any increase in activity due to the introduction of a methyl radical into the aromatic nucleus of these quaternary compounds. Although each of the products in this group showed definite germicidal activity, in general the introduction of the methyl group was not accompanied by any significant increase in such activity.

REFERENCES

(1) Domagk, G., *Deut. Med. Wochschr.*, 61 (1935), 829.

(2) Coulthard, C. E., Marshall, J., and Pyman, F. L., *J. Chem. Soc.* (1930), p. 280.

(3) Muldoon, H. C., "A Textbook of Organic Chemistry," Second Edition (1936), p. 420.

(4) Knight, G. A., and Shaw, B. D., *J. Chem. Soc.* (1938), p. 682.

(5) Ruehle, G. L. A., and Brewer, C. M., U. S. Dept. Agriculture Circular No. 198 (1931), p. 4.

(6) Kolloff, H. G., and Hunter, J. H., *J. Am. Chem. Soc.*, 63 (1941), 490.

(7) Norris, J. F., and Taylor, H. B., *Ibid.*, 46 (1924), 753; also "Organic Syntheses," Collective Vol. 1 (1932), 137.

(8) Levene, P. A., and West, C. J., *J. Biol. Chem.*, 18 (1914), 478.

(9) Chemische Fabrik Von Heyden A.-G., French Patent 812,360 (May 8, 1937); cf. *Chem. Abstr.*, 34 (1930), 7068.

# The Influence of Aloe and Podophyllum on the Flow of Hepatic Bile in the Dog*

*By Lloyd W. Hazleton*†

Recent investigations (1, 2) indicate that acute fistula methods for studying the flow of hepatic bile give reliable results if conditions are properly controlled. The work of Kocour and Ivy (3) establishes the uniform production of bile by the liver in dogs with chronic biliary fistulas in which the gall bladder is excluded and the bile collected under suction at all times.

The following investigations on the choleretic properties of Extract of Aloe (N. F. VI) and of Resin of Podophyllum (U. S. P. XI) employ the acute technique of Co Tui (2) with certain modifications.

Table I.—The Effect of Extract of Aloe and Resin of Podophyllum on the Flow of Hepatic Bile and Blood Pressure of Dogs

| Dog No. | Drug[a] | Choleretic Dose, Mg. | Route | Bile, Average per Period, Cc. Control | Bile, Average per Period, Cc. Exptl. | Blood Pressure, Mm. Hg Control | Blood Pressure, Mm. Hg Exptl. |
|---|---|---|---|---|---|---|---|
| 3 | C | ... | .. | 0.7 | 0.5[b] | ... | ... |
| 4 | C | ... | .. | 1.5 | 1.5 | ... | ... |
| 13 | C | ... | .. | 1.0 | 1.4 | 80–166 | 68–104 |
| 24 | C | ... | .. | 0.8 | 1.4 | 96–137 | 146 |
| 32 | C | ... | .. | 1.8 | 2.8 | 108–160 | 108–116 |
| | | Total | | | | | |
| 2 | P | 100 | Tube | 0.7 | 1.2 | ... | ... |
| 5 | P | 100 | Tube | 1.0 | 1.1 | ... | ... |
| 6 | P | 100 | Tube | 1.7 | 1.4 | ... | ... |
| 7 | P | 100 | Tube | 2.0 | 2.2 | ... | ... |
| 26 | P + A | 50 Each | Tube | 1.4 | 0.8 | 140–174 | 112–160 |
| 28 | P + A | 50 Each | Tube | 1.7 | 2.7 | 106–135 | 110–124 |
| | | per Kg. | | | | | |
| 9 | A | 5 | Vein | 0.7 | 1.4 | ... | ... |
| 11 | A | 5 | Vein | 1.5 | 2.0 | 138 | 138–150 |
| 14 | A | 5 | Vein | 0.6 | 0.9 | 160 | 150 |
| 15 | A | 5 | Vein | 2.1 | 2.4 | 160–170 | 160–172 |
| 18 | P | 5 | Vein | 1.0 | 2.0 | 144–188 | 140–166 |
| 20 | P | 5 | Vein | 1.2 | 2.2 | 148–166 | 150–200 |
| 34 | P | 2 | Vein | 3.5 | 5.5 | 98–114 | 108–138 |
| 36 | P | 2 | Vein | 1.6 | 3.2 | 148 | 156–165 |
| 38 | P | 2 | Vein | 1.6 | 3.2 | 132–154 | 134–164 |
| 62 | R | 2 | Vein | 1.6 | 1.8 | 130 | 135 |
| 68 | R | 5 | Vein | 1.3 | 1.6 | 145 | 158 |

[a] C = control, no drug; P = resin of podophyllum; A = extract of aloe; R = rosin.
[b] Experimental interval in control animals corresponds to interval following administration of drug to treated animals.

## EXPERIMENTAL

Adult dogs, fasted over night and anesthetized with 30 mg. per Kg. of sodium pentobarbital administered intravenously, were used in these ex-

* From the Henry E. Kalusowski Memorial Laboratory, The George Washington University School of Pharmacy, Washington, D. C.

Acknowledgment is made of a grant from the Proprietary Association in support of this study.

† Assistant Professor of Pharmacology.

periments. The number of male and female animals was approximately equal and no distinction was made between the two. A midline incision was made and the cystic duct tied or clamped to eliminate bladder bile, and the common duct was cannulated with a glass cannula connected to gum tubing. The tubing was exteriorized through the incision and the opening adjusted to the level of the common duct to eliminate hydrostatic pressure.

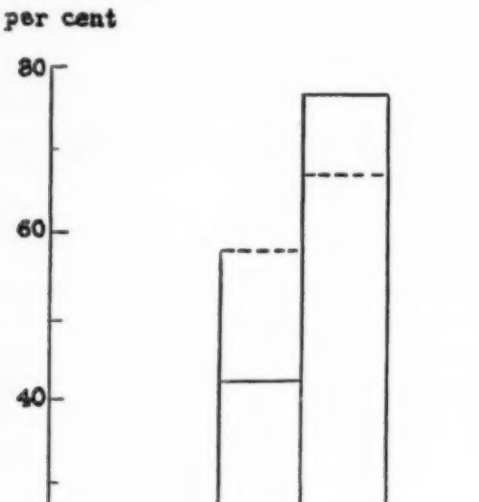



Fig. 1.—The Per Cent of Increase in Biliary Flow Following the Administration of Drugs, and During a Comparable Interval in Control Animals.

C = control, no drug, 5 dogs; T = resin of podophyllum alone or with extract of aloe given by intestinal tube, 6 dogs; A = extract of aloe intravenously, 4 dogs; P = resin of podophyllum intravenously, 5 dogs; R = rosin intravenously, 2 dogs. Dotted line indicates value when additional periods are included.

Tracheal cannulas were routinely inserted and the femoral vein exposed for injection. In all but the earlier experiments blood pressure tracings were made from the cannulated carotid artery and respiratory tracings were taken from the tracheal cannula. Additional sodium pentobarbital was administered as needed to maintain uniform anesthesia. The bile was collected in graduated tubes and measured at the end of each 30-minute period.

*Controls.*—To establish the rate of biliary flow throughout the duration of the experiments (about four hours), five dogs which received no drug other than the anesthetic were used as controls. The averages of the first four and of the second four periods in the individual animals are recorded in Table I. These correspond, respectively, to the control and experimental intervals described below. The percentage increase in biliary flow for the second interval is shown in Fig. 1. Three of these experiments were continued through additional periods, but there was no change in the average figures.

Due to individual variation in the above animals, the rate of flow was established for each dog over a period of four 30-minute periods prior to the administration of the drug. The values given in Table I are the averages for the first four periods (control interval) and the four periods (experimental interval) immediately following the administration of aloe, podophyllum or rosin. Percentage changes from the average control values are recorded in Fig. 1.

*Duodenal Administration.*—In six of the dogs drugs were administered into the duodenum by means of a catheter inserted through a stab wound in the pylorus. Each of four dogs received 100 mg. of resin of podophyllum (total). The remaining two received 50 mg. of resin of podophyllum plus 50 mg. of extract of aloe. The results are shown in Table I and the average percentage changes in Fig. 1.

*Intravenous Administration.*—Four dogs were given intravenous injections of 5 mg. per Kg. of extract of aloe in aqueous solution. The results are given in Table I and Fig. 1. Three experiments were conducted for additional periods and the dotted area of Fig. 1 shows the effect of including these additional periods.

Two dogs received 5 mg. per Kg. of resin of podophyllum which was dissolved by the addition of a sufficient quantity of 0.5*N* potassium hydroxide and diluted to 5 cc. Three additional animals received 2 mg. per Kg. of resin of podophyllum dissolved in alcohol and diluted to 40% alcoholic concentration by the addition of 0.2*M* sodium phosphate. The results are given in Table I and Fig. 1.

One dog was given 2 mg. and another 5 mg. per Kg. of Rosin (U. S. P. XI) intravenously. The rosin was boiled in distilled water for one-half hour, dried, weighed, dissolved in alcohol and the solution diluted to 40% alcoholic concentration by the addition of 0.1*M* sodium phosphate. The results are given in Table I and Fig. 1.

*Effect of Blood Pressure.*—In the course of the experiments four dogs developed blood pressures of below 60 mm. of mercury after an initial level of 100 mm. or above. These were allowed to remain uncorrected and the flow of bile observed. Following the drop in pressure the biliary flow decreased to such an extent that the average for the four animals was only 0.4 cc. per period. In one animal with a mean pressure of 58 mm. of mercury induced by hyper-ventilation there was a cessation of biliary flow for one period (30 minutes). Upon elevation of

the mean pressure to 84 mm. by additional anesthetic, the flow increased to above 2.0 cc. per period and remained at this level for the duration of the experiment. The results from these animals were deleted from the series and do not appear in the table or figure.

## DISCUSSION

Although Co Tui (2) found that under conditions similar to the above the flow of bile in dogs reached a constant level or decreased during the control period, and Halpert (4) reported similar results in rabbits, the results presented above indicate that in dogs there is a tendency toward an increase in volume during the later periods. This phenomenon must be taken into consideration when evaluating the choleretic properties of a drug, as three of the five control dogs in this series showed an increase, the average being 25%. Markowitz (5), using ether anesthesia, observed a marked decrease or cessation of biliary flow for 24 hours after the operation, but Coffey, *et al.* (6), could detect no reduction in biliary flow following the intravenous administration of sodium amytal to a chronic fistula dog.

The administration of resin of podophyllum alone or in combination with extract of aloe by duodenal tube resulted in values which were too irregular to interpret accurately, probably due in part to variability in absorption during anesthesia and the limited period of the experiments. In two of the six experiments the flow was decreased, while the average increase in volume for the group was approximately 7%.

The intravenous injection of extract of aloe was followed by a 42% increase in the average bile production within the experimental interval of four 30-minute periods. In the three experiments which were prolonged for three or four 30-minute periods beyond the usual experimental interval the rate of biliary flow continued to increase or remained at a high level. Inclusion of these results brings the average increase over the control value to 58%, as indicated by the dotted line in Fig. 1. The inclusion of additional periods in the control animals did not change the per cent of increase. From these observations it is apparent that the choleretic action of extract of aloe is comparatively slow in onset and of long duration. In contrast to the above two groups there was no incidence of decrease in bile production and the smallest increase was 14%. The injections were followed by a slight increase in blood pressure with no untoward effects.

Intravenous injection of resin of podophyllum produced a 77% increase in the average bile production. The smallest individual increase was 57%. Inclusion of results from the two experiments which were continued for additional periods gives an average increase of 67%, indicating that the maximum action was reached within the four 30-minute periods of the standard experimental interval. The rate of flow during the control interval (four 30-minute periods) does not seem to be a factor in this choleretic effect (see dog No. 34, Table I). The injections were followed by a gradual increase in blood pressure accompanied by strong pulsations and an occasional extra systole. The condition receded and usually disappeared within about one hour. Since the increase in blood pressure is not proportional to the increase in bile, and there is no other apparent correlation between these two factors except in cases of extremely low pressure, it is apparent that the increased choleresis is not a result of the increase in blood pressure. Stewart and Ryan (7) observed in clinical cholecystographic studies that four hours after the administration of podophyllin (Resin of Podophyllum) the gall bladder was very much more distended with bile than before the drug was given.

Since intravenous injection of rosin increases the flow of bile only 13%, and this is somewhat less than the normal increase during a comparable period in control animals, it is apparent that the choleretic action of extract of aloe and of resin or podophyllum is not a non-specific phenomenon due to foreign or insoluble substances.

The observations made on the effect of low blood pressure indicate that this may be a source of error in acute studies where blood pressure has not been recorded. Values below 60 mm. of mercury may be sufficient for maintenance of the anesthetized animals, but lead to rapid decrease or cessation of the flow of hepatic bile. In one animal

correction of hypotension was followed by a marked increase in bile production.

### CONCLUSIONS

Intravenous injection of extract of aloe increases the flow of hepatic bile in the anesthetized dog. The duration of action is relatively long.

Intravenous injection of resin of podophyllum increases the flow of hepatic bile in the anesthetized dog. This action apparently reaches a maximum within two hours after the injection.

Intravenous injection of rosin has no effect upon the biliary flow under the above conditions.

There is a critical level of blood pressure necessary to the maintenance of biliary flow. Evidence presented indicates this to be approximately 60 mm. of mercury for the dog.

### REFERENCES

(1) Chabrol, E., and Charronnat, R., *Paris Med.*, 19 (1929), 509.

(2) Co Tui, F. W., *J. Lab. Clin. Med.*, 19 (1934), 564.

(3) Kocour, E. J., and Ivy, A. C., *Am. J. Physiol.*, 122 (1938), 325.

(4) Halpert, B., *Proc. Soc. Exptl. Biol. Med.*, 39 (1938), 115.

(5) Markowitz, J., "Textbook of Experimental Surgery" (1937), W. Wood and Company, Baltimore, Md.

(6) Coffey, R. J., Koppanyi, T., and Linegar, C. R., *Am. J. Digestive Dis.*, 7 (1940), 21.

(7) Stewart, W. H., and Ryan, E. J., *Am. J. Roentgenol.*, 19 (1928), 341.

---

# The Assay of Yellow Mercuric Oxide Ointment, U. S. P. XI*

*By F. N. Van Deripe and R. A. Konnerth†*

The U. S. P. XI assay method for mercury in Ointment Mercury Oxide has given satisfactory results when used on freshly prepared ointments, but not on aged samples. With the latter the figures are lower than those obtained initially on the same samples. This is not due to any loss of mercury from the ointment, but presumably to some change whereby the mercury becomes combined in some form which is unavailable under the conditions of the U. S. P. XI assay. For example, it might gradually react with acids in the ointment base to form mercury soaps in which the mercury is no longer available in so far as the U. S. P. XI assay is concerned. That this is the case has been indicated by the fact that aged samples with a low U. S. P. XI assay value have given results close to the original ones when a somewhat different analytical method (1) is employed whereby the entire mercury content of the sample is determined. These findings show not only that the U. S. P. XI method may with aged samples give results lower than the total mercury present, but also that upon aging changes do occur slowly whereby a small portion of the mercury in this ointment enters into a different state of chemical combination.

### EXPERIMENTAL

A laboratory preparation of Ointment Mercury Oxide Yellow, U. S. P. XI, showing 1.01% mercuric oxide by the U. S. P. XI method and 1.03% by the method described below was stored at room temperature and 105° F. for periodic assay by both methods.

*Assay.*—Weigh accurately (within 0.0005 Gm.) about 2.5 Gm. of the sample and transfer into a dry beaker of about 250 cc. capacity. Dissolve directly by warming on a water bath to 50° C. with 100 cc. of a solvent mixture consisting of benzene, 13 parts by volume, acetic acid (glacial), 2 parts, and alcohol

---

* Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.

† From the Analytical Department of the Pharmaceutical and Chemical Laboratories, E. R. Squibb & Sons, Brooklyn, N. Y.

Table I

| | Initial Assay, Per Cent | Storage Conditions | Reassays after Storage, Per Cent ½ Mo. | 1½ Mo. | 13½ Mo. | 25½ Mo. |
|---|---|---|---|---|---|---|
| U. S. P. XI method | 1.01 | Room temp. | 1.02 | 1.01 | 0.92 | 0.86 |
| | | 105° F. | .. | 1.04 | 0.83 | .. |
| Experimental method | 1.03 | Room temp. | 1.02 | 1.01 | 1.02 | 1.00 |
| | | 105° F. | .. | 1.01 | 1.00 | .. |

(90%), 5 parts. The resulting clear solution is treated with a rapid stream of hydrogen sulfide gas to precipitate the mercury; precipitation is complete within 10 minutes and the mercuric sulfide settles quickly in a granular form. Warm the beaker and its contents to about 50° C. Collect the mercuric sulfide on a dried, weighed, asbestos-prepared Gooch crucible using a filter pump. Rinse the hydrogen sulfide inlet tube, the beaker and the precipitate on the Gooch with hot benzene and then with a little alcohol. Any particles of mercuric sulfide adhering to the walls of the beaker are removed and transferred to the filter by a final rinse with warm water. Dry the precipitate to constant weight at 120° C. and weigh accurately (within 0.0005 Gm.). The weight of mercuric sulfide found multiplied by 0.9310 equals the weight of mercuric oxide.

### DISCUSSION

The results are shown in Table I.

This tabulation shows clearly that after a period of approximately a year the U. S. P. XI method does not determine all of the mercury.

In addition it may be noted here that the experimental method offers greater ease of manipulation. There is some difficulty encountered in the U. S. P. XI method at the very beginning where the 10-Gm. sample has to be introduced into a separatory funnel and then mixed with the ether. Later after the hydrochloric acid and water have been added, vigorous shaking is directed to dissolve the mercuric oxide. This, in our hands, has resulted in emulsions which sometimes resist separation and, instead, forms a gelatinous intermediate layer interfering with the subsequent filtration and making the washing and draining long and tedious.

### CONCLUSION

The modified assay method described herein measures the total mercury content of Ointment Mercury Oxide Yellow, U. S. P. XI, even after aging and even though some of the mercury oxide has undergone change to form something in which the mercury is in a different state of combination. It is superior to the U. S. P. XI method in speed and simplicity.

### REFERENCE

(1) Green, L. W., and Schoetzow, R. E., JOUR. A. PH. A., 19 (1930), 471.

---

# Preliminary Antispasmodic Tests of a Series of Morpholino and Some Other Compounds*

*By L. W. Rowe†*

For more than two years a group of morpholino and some other compounds, which were synthesized by research chemists in our laboratories (1), have been subjected to preliminary pharmacological tests in an effort to select a very limited number of the most promising for more thorough investigation. The results of the detailed study on a more limited number of compounds will be reported later by another group (2).

The method used for determining relative antispasmodic action was that of Magnus (3, 4) in which excised intestinal strips from guinea pigs or rabbits were suspended in warm, oxygenated Locke-Ringer Solution and exposed alternately to definite concentrations of the unknowns and of

* Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.

† From the Research Laboratories of Parke, Davis and Co., Detroit, Mich.

papaverine hydrochloride or diphenylacetyl-diethyl-aminoethanol hydrochloride (Trasentin) which were both used as controls. The comparative relaxing action was determined directly on the same and different strips. Some work was done with the antagonistic effect against barium chloride but this will not be included in the report.

The relative toxicity was determined upon white mice dosed intraperitoneally by finding the amount of each compound in milligrams per gram which would produce a 60% mortality.

In the first table only the morpholino compounds will be given, together with the short symbol by which they will be referred to later and their toxicity to white mice.

In the second table compounds other than morpholines together with the short symbol by which they will be referred to and their toxicity to white mice are listed.

**Table I**

| | Compound | Symbol | M. L. D., Mg. per Gm. |
|---|---|---|---|
| 1. | *N*-β-Morpholine-ethyl diphenylchloroacetamide HCl | S-2 | 0.50 |
| 2. | β-4-Morpholine-ethyl diphenylacetate HCl | S-5 | 0.80 |
| 3. | Diphenyl acetic acid β-morpholine-ethyl amide HCl | S-6 | 0.30 |
| 4. | β-4-Morpholine-ethyl cinnamate HCl | S-8 | 1.50 |
| 5. | γ-4-Morpholine-propyl diphenylacetate HCl | S-9 | 0.35 |
| 6. | β-4-Morpholine-ethyl benzilate HCl | S-10 | 0.30 |
| 7. | ε-4-Morpholine-butyl diphenylacetate HCl | S-11 | 0.30 |
| 8. | β-Methyl-β-4-morpholine-propyldiphenylacetate HCl | S-12 | 0.70 |
| 9. | β-4-Morpholine-ethyl triphenylacetate HCl | S-13 | 0.40 |
| 10. | β-4-Morpholine-ethyl diphenylchloroacetate HCl | S-14 | 0.20 |
| 11. | γ-4-Morpholine-propyl diphenylacetate bromobenzylate | S-15 | 0.04 |
| 12. | β-4-Morpholine-ethyl dibenzylacetate HCl | S-16 | 0.60 |
| 13. | γ-Methyl-β-4-morpholine-ethyl diphenylacetate HCl | S-17 | 0.10 |
| 14. | β-4-Morpholine-ethyl cyclohexane carboxylate HCl | S-18 | 2.50 |
| 15. | β-Methyl-β-4-morpholine-1-propanol | S-20 | 0.70 |
| 16. | β-4-Morpholine-ethyl tertiary butylacetate HCl | S-22 | 1.0 |
| 17. | β-4-Morpholine-ethyl trimethylacetate sulfate | S-23 | 1.25 |
| 18. | β-4-Morpholine-ethyl phenylacetate HCl | S-24 | 1.50 |
| 19. | β-4-Morpholine-ethyl diphenyl propionate HCl | S-25 | 0.50 |
| 20. | β-4-Morpholine-ethyl-2-camphane carboxylate HCl | S-26 | 0.35 |
| 21. | Dimethyl-4-morpholine-propyl diphenylacetate HCl | S-28 | 1.20 |
| 22. | 4-Morpholine-*N*-hexyl diphenylacetate HCl | S-29 | 0.40 |
| 23. | β-4-Morpholine-ethyl diphenylacetate HBr | S-30 | 0.80 |
| 24. | β-4-Morpholine-ethyl *N,N*-diphenylcarbamate HCl | S-32 | 0.55 |
| 25. | β-4-Morpholine-ethyl phenylcyclohexaneacetate HCl | S-35 | 0.40 |
| 26. | Phenylcyclohexaneacetate acid β-4-Morpholine ethylamide HCl | S-36 | 0.30 |
| 27. | Dimethyl-4-morpholine-propyl phenylcyclohexaneacetate HCl | S-37 | 0.50 |
| 28. | β-4-Morpholine-ethyl acetoxydiphenylacetate HCl | S-38 | 0.30 |
| 29. | Dimethyl-4-morpholine-propyl diphenylacetate sulfate | S-39 | 1.0 |
| 30. | Dimethyl-4-morpholine-propyl diphenylacetate benzyl hydrobromide | S-40 | 1.1 |
| 31. | Dimethyl-4-morpholine-propylbenzaote HCl | S-41 | 0.9 |
| 32. | Dimethyl-4-morpholine-propylcinnamate HCl | S-42 | 1.0 |

**Table II**

| | Compound | Formula | Symbol | M. L. D., Mg. per Gm. |
|---|---|---|---|---|
| 1. | Papaverine HCl | $C_{20}H_{21}O_4N.HCl$ | Papaverine | 0.15 |
| 2. | Diphenylacetyl-diethylaminoethanol HCl | $C_{20}H_{23}O_2N.HCl$ | Trasentin | 0.35 |
| 3. | Sodium diphenyl hydantoinate | $C_{15}H_{11}O_2N_2.Na$ | Dilantin | 0.15 |
| 4. | β-Diethylaminoethyl benzilate HCl | $C_{20}H_{25}O_3N.HCl$ | S-1 | 0.10 |
| 5. | β-Diethylaminoethyl diphenylchloroacetate HCl | $C_{20}H_{24}O_2NCl.HCl$ | S-3 | 0.18 |
| 6. | γ-Diethylaminopropyl diphenylchloroacetate HCl | $C_{21}H_{26}O_2NCl.HCl$ | S-4 | 0.06 |
| 7. | *N*-Methyl cyclohexylamine HCl | $C_7H_{15}N.HCl$ | S-7 | 0.40 |
| 8. | Diethylamino-hydroxy propyl diphenylacetate HCl | $C_{21}H_{27}O_3N.HCl$ | S-27 | 0.18 |
| 9. | Dimethyl butyrylamino-pyridine HCl | $C_{11}H_{16}ON_2.HCl$ | S-31 | 0.60 |
| 10. | β-Diphenyl acetamino-pyridine HCl | $C_{19}H_{16}ON_2.HCl$ | S-33 | 0.40 |
| 11. | β-Ethyl phenylamino-ethyldiphenylacetate HCl | $C_{24}H_{25}O_2N.HCl$ | S-34 | 0.40 |
| 12. | Methyl phenylpiperidine carbonic acid ethyl ester | $C_{16}H_{21}O_2N$ | S-140 | 1.75 |

Table III.

| Compound | Antispasmodic Activity, Per Cent of Papaverine | Effective Dilutions |
|---|---|---|
| 1. S-2 | 50 | 1- 25,000 to 1- 50,000 |
| 2. S-5 | 75 | 1- 35,000 to 1- 75,000 |
| 3. S-6 | 20 | 1- 10,000 to 1- 20,000 |
| 4. S-8 | 40 | 1- 20,000 to 1- 40,000 |
| 5. S-9 | 75 | 1- 35,000 to 1- 75,000 |
| 6. S-10 | 25 | 1- 12,500 to 1- 25,000 |
| 7. S-11 | 60 | 1- 30,000 to 1- 60,000 |
| 8. S-12 | 60 | 1- 30,000 to 1- 60,000 |
| 9. S-13 | 25 | 1- 12,500 to 1- 25,000 |
| 10. S-14 | 75 | 1- 35,000 to 1- 75,000 |
| 11. S-15 | 50 | 1- 25,000 to 1- 50,000 |
| 12. S-16 | 60 | 1- 30,000 to 1- 60,000 |
| 13. S-17 | 60 | 1- 30,000 to 1- 60,000 |
| 14. S-18 | 10-20 | 1- 5,000 to 1- 20,000 |
| 15. S-20 | 5-10 | 1- 2,500 to 1- 10,000 |
| 16. S-22 | 5-10 | 1- 2,500 to 1- 10,000 |
| 17. S-23 | 5 | 1- 2,500 to 1- 5,000 |
| 18. S-24 | 10 | 1- 5,000 to 1- 10,000 |
| 19. S-25 | 50 | 1- 25,000 to 1- 50,000 |
| 20. S-26 | 60 | 1- 30,000 to 1- 60,000 |
| 21. S-28 | 200 | 1-100,000 to 1-200,000 |
| 22. S-29 | 150 | 1- 75,000 to 1-150,000 |
| 23. S-30 | 40 | 1- 20,000 to 1- 40,000 |
| 24. S-32 | 30 | 1- 15,000 to 1- 30,000 |
| 25. S-35 | 100 | 1- 50,000 to 1-100,000 |
| 26. S-36 | 50 | 1- 25,000 to 1- 50,000 |
| 27. S-37 | 10 | 1- 5,000 to 1- 10,000 |
| 28. S-38 | 25 | 1- 12,500 to 1- 25,000 |
| 29. S-39 | 200 | 1-100,000 to 1-200,000 |
| 30. S-40 | 100 | 1- 50,000 to 1-100,000 |
| 31. S-41 | 40 | 1- 20,000 to 1- 40,000 |
| 32. S-42 | 40 | 1- 20,000 to 1- 40,000 |

Table IV

| Compound | Antispasmodic Activity, Per Cent of Papaverine | Effective Dilution |
|---|---|---|
| 1. Papaverine | 100 | 1-50,000 to 1-100,000 |
| 2. Trasentin | 100 | 1-50,000 to 1-100,000 |
| 3. Dilantin | 25 | 1-12,500 to 1- 25,000 |
| 4. S-1 | 50 | 1-25,000 to 1- 50,000 |
| 5. S-3 | 50 | 1-25,000 to 1- 50,000 |
| 6. S-4 | 75 | 1-35,000 to 1- 75,000 |
| 7. S-7 | 10 | 1- 5,000 to 1- 10,000 |
| 8. S-27 | 30 | 1-15,000 to 1- 30,000 |
| 9. S-31 | 20 | 1-10,000 to 1- 20,000 |
| 10. S-33 | 10 | 1- 5,000 to 1- 10,000 |
| 11. S-34 | 10 | 1- 5,000 to 1- 10,000 |
| 12. S-140 | 5 | 1- 2,500 to 1- 5,000 |

In Tables III and IV the relative antispasmodic activity of the compounds listed in Tables I and II, respectively, are given in terms of papaverine hydrochloride as the control or 100%. In most instances these percentages are the averages of several days' comparisons on a series of five or six intestinal strips each day. After each strip was subjected to a definite dilution of the drug until the maximum action of the dose was reached, the solution was withdrawn and fresh, warm Locke-Ringer Solution was added. The variation in dilution noted in the table was that used on different strips and on different days.

## DISCUSSION

In the entire series S-28 and S-39 show up best and these are merely different salts of the same compound. Their activity by the Magnus method is fully twice that of papaverine hydrochloride and their toxicity is only about one-eighth or 12.5% as great. S-29, S-35 and S-40 are also quite active, but in the case of the first two the toxicities are somewhat nearer that of papaverine. Then there is a group of four compounds S-4, S-5, S-9 and S-14 whose activities are about three-fourths that of papaverine, but only S-5 of this group has a low toxicity.

Dilantin which is primarily a good anticonvulsant, is shown to have a rather weak antispasmodic action by the method used, namely, about 25% that of papaverine.

## SUMMARY

1. One morpholine compound, dimethyl-4-morpholine propyl diphenylacetate, was found to be the most promising in the preliminary tests of a series of 32 experimental morpholine preparations.

2. Several others were found to be fully as good as papaverine but not as active as S-28.

3. Dilantin showed a definite but relatively weak antispasmodic action.

## REFERENCES

(1) Bywater, Cheney, *et al.*, *J. Am. Chem. Soc.* (in press).

(2) Yonkman, Lehman and Chase, *J. Pharmacol.* (in press).

(3) Magnus, *Arch. ges. Physiol.*, 102 (1904), 123, 349.

(4) *Ibid.*, 103 (1904), 515.

# An Evaluation of the Cathartic Action of Bile Salts Following Acute and Chronic Administration to Mice*

*By Lloyd W. Hazleton and Frank Fortunato†*

A survey of the literature regarding the cathartic action of bile and bile salts reveals that most of the observations are based on acute experiments. Fubini and Luzzati (1) found that bile increases peristaltic movements of the small intestine, and Horrall, *et al.* (2), observed that bile and bile salts have a temporary local stimulant effect on the motility of the small intestine in anesthetized dogs. According to Haney, *et al.* (3), the application of bile to the mucous surface of the small intestine of dogs results in an increase of propulsive motility which is attributed to the influence of bile salts. Van Liere and Northup (4) reported a decrease in the emptying time of the stomach in humans following administration of bile acids. Broun, *et al.* (5), observed that large amounts of dog bile act as a purgative following oral administration to dogs. Whipple and Smith (6) found an increase in bile salt secretion following daily bile feeding to dogs, and they observed diarrhea in the animals which received large quantities (21 Gm.) of bile by mouth.

The present investigations are based on the effect of acute and chronic administrations of bile salts to mice. With slight modifications, the method for the bioassay of senna described by Geiger (7) was adapted to this procedure.

## EXPERIMENTAL

Geiger's method consists of the bioassay of cathartic drugs in mice by the following procedure. After expression of urine from the bladder, the animals are placed in individual one-liter beakers,[1] the bottoms of which are covered with a double layer of filter paper held in place by a piece of one-third inch wire mesh. Food is supplied during the course of the experiment. After observation for about one-half hour, those mice whose stools are sufficiently moist to stain the filter paper are rejected. The balance are suitable for the evaluation of cathartic action in the following manner.

A solution of the drug is administered by stomach tube in 0.5-cc. volumes, using 16 or 18 gage hypodermic needles to the blunted end of which is fixed a small tear drop of solder. After replacing the mice in the beakers the stools are observed at regular intervals. Stools sufficiently soft to moisten the paper and leave a clearly visible stain are an indication of catharsis and are designated as a positive action.

Preliminary experiments confirmed Geiger's observation that 0.5 cc. of distilled water, *per se*, did not cause catharsis.

The term "bile salts," as used in this report, designates a product commercially known as Bile Salts Compound,[2] which contains not less than 75% of the following combined salts: sodium glycocholate, sodium glycodesoxycholate, sodium glycohyodesoxycholate, sodium taurocholate, sodium taurodesoxycholate and sodium taurohydrodesoxycholate. The remainder consists mainly of inorganic matter and salts of free fatty acids present in bile. In addition, there may also be included small amounts of protein, fat, pigment and other constituents of normal bile.

Stock aqueous mixtures containing 80 mg. of bile salts per cc. were prepared and all other concentrations made from these by dilution with distilled water.

The temperature of the animal room should be kept above 20° C., since lower temperature are likely to cause diarrhea (7).

All animals were given Purina Dog Chow Checkers and water, *ad lib.* The cages were constructed with wire mesh bottoms, and to permit daily gross observation of the nature of the stools, the dropping pan was covered with paper.

A group of young male mice was used in preliminary studies to determine the effectiveness of bile salts in producing catharsis. Attempts in this direction, using small doses (2 and 4 mg.) and observing the animals for a period of one hour, were unsuccessful. However, chronic administration of 2 mg. of bile salts per mouse per day was begun in

* Acknowledgment is made of a grant from the Proprietary Association in support of this study.

Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.

† From the Henry E. Kalusowski Memorial Laboratory for Pharmacological Research, The George Washington University School of Pharmacy, Washington, D. C.

[1] In this laboratory it was found very convenient to employ frog assay tank compartments elevated about one-half inch above the filter paper.

[2] Acknowledgment is made of the courtesy of the American Ferment Company in supplying this drug.

Table I.—Determination of Threshold Cathartic Dose for Bile Salts in Adult Mice

| No. Mice | Bile Salts Mg./Mouse | Results 1st Hr. + | 1st Hr. − | 2nd Hr. + | 2nd Hr. − | 3rd Hr. + | 3rd Hr. − | 4th Hr. + | 4th Hr. − |
|---|---|---|---|---|---|---|---|---|---|
| 34[a] | 4 | 7[b] | 27 | .. | .. | .. | .. | .. | .. |
| 5 | 10 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 |
| 11 | 15 | 1 | 10 | 1 | 10 | 1 | 10 | 1 | 10 |
| 9 | 30 | 0 | 9 | 1 | 8 | 2 | 7 | 2 | 7 |
| 8 | 40 | 0 | 8 | 2 | 6 | 3 | 5 | 4 | 4 |

[a] Young male mice, all others adult.
[b] Observation period one hour.

Table II.—Results of Assays in Control and Bile-Salt Treated Mice at Approximately Thirty-Day Intervals

| No. Mice | Daily Bile Salts, Mg./Mouse | Chronic Administration, Days | Assay Dose, Mg./Mouse | Results 1st Hr. + | 1st Hr. − | 2nd Hr. + | 2nd Hr. − | 3rd Hr. + | 3rd Hr. − | 4th Hr. + | 4th Hr. − | Positive, Per Cent |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 28 | 2–30 days | | | | | | | | | | | |
| | 4–30 days | 60 | 40 | 13 | 16 | 20 | 8 | 25 | 3 | 27 | 1 | 96 |
| 25 | Controls | 60 | 40 | 7 | 18 | 11 | 14 | 15 | 10 | 15 | 10 | 60 |
| 22 | 20 | 90 | 30 | 7 | 15 | 13 | 9 | 16 | 6 | 18 | 4 | 82 |
| 27 | Controls | 90 | 40 | 9 | 18 | 18 | 9 | 20 | 7 | 23 | 4 | 85 |
| 22 | 20 | 120 | 30 | 9 | 13 | 15 | 7 | 18 | 4 | 19 | 3 | 86 |
| 20 | Controls | 120 | 40 | 6 | 14 | 9 | 11 | 10 | 10 | 11 | 9 | 55 |
| 19 | 20 | 157 | 40 | 12 | 7 | 15 | 4 | 17 | 2 | 18 | 1 | 94 |
| 24 | Controls | 154 | 30 | 8 | 16 | 14 | 10 | 16 | 8 | 17 | 7 | 70 |
| 20 | 0–8 days | 165 | 30 | 5 | 15 | 6 | 14 | 11 | 9 | 11 | 9 | 55 |
| 20 | Controls | 165 | 30 | 0 | 20 | 3 | 17 | 5 | 15 | 6 | 14 | 30 |

a group of 30 young male mice, with a comparable number of animals serving as controls.

In view of the ineffectiveness of small doses of bile salts, the original procedure based on senna was duplicated for further study of the method. One-half cc. volumes of a 5% infusion of senna were administered to each of 15 additional mice which were observed for a period of two hours. At the end of 30 minutes two were positive, at one hour four were positive, while after two hours 14 were positive. The fluidity of the stools at this time was so marked that readings could be made at a glance. In view of the difference between these results and those obtained previously with bile salts, the action of bile salts was reinvestigated using larger doses and longer periods of observation. Under these conditions a degree of catharsis comparable to that induced with senna was obtained, thus making it possible to establish a T. C. D. (threshold cathartic dose—the dose which will produce catharsis in approximately 50% of the animals) for bile salts. Although the animals were supplied with water and left over night, it was observed that no further positive results were obtained after four hours, and this was accepted as a suitable period of observation. The results of these determinations are summarized in Table I. Based upon these experiments, 40 mg. was selected as the T. C. D. for the assay at the end of the 60-day period of chronic administration. This group of animals had received daily doses of 2 mg. for 30 days and 4 mg. for 30 days. The last daily dose was administered approximately 24 hours before the assay period. The results are shown in Table II. It will be noted that cathartic action occurred in 96% of the mice which had received daily doses of bile salts and in only 60% of the controls, which receive no daily bile salts.

The experimental animals were continued for an additional period of 30 days (90 days, total) using a daily dose of bile salts representing approximately one-half the T. C. D., *i. e.*, 20 mg. per mouse. At the end of this time the T. C. D. (40 mg.) was given to the controls. The animals which received the daily dose of bile salts were given 30 mg. per mouse instead of 40 mg. as in the previous assay. The results are summarized in Table II.

Following another 30-day period (120 days, total), during which the experimental group of mice continued to receive the daily dose (20 mg.), the assay was repeated as above: 30 mg. to experimental animals, 40 mg. to controls. Table II summarizes the results of this assay. As indicated in the 90-day assays the per cent of positive results for the experimental animals (82% at 30 mg.) and for the controls (85% at 40 mg.) closely approximate each other. In the 120-day assay, however, there is a greater difference in the per cent of positive actions between the experimental animals (86% at 30 mg.) and the controls (55% at 40 mg.). The average weight of experimental and control animals at this time was 20.0 Gm. and 24.0 Gm., respectively.

Daily administration of bile salts (20 mg.) was continued for another period of 30 days (150 days, total). At the end of this time the assay was repeated using 0.5-cc. volumes of 1:15 dilution (the approximate T. C. D., see Table III) of fluidextract of senna instead of bile salts. From the results obtained (Fig. 1 and Table III) it is apparent, within the time limitations of the test, that (*a*) the onset of active catharsis from comparable doses of bile salts is more rapid than that from senna, and (*b*) the cathartic effect of senna remains comparatively unchanged as a result of chronic administration of bile salts to this group of mice. It should be

Table III.—Determination of the Approximate T. C. D. of Fluidextract of Senna, in 0.5-Cc. Doses, and the Effect of Daily Oral Administration of Bile Salts upon its Cathartic Action in Mice

| No. Mice | Conc. of Fldext. | Bile Salts Daily Dose in Mg. | No. of Days | Results 1st Hr. + | 1st Hr. − | 2nd Hr. + | 2nd Hr. − | 3rd Hr. + | 3rd Hr. − | 4th Hr. + | 4th Hr. − | Positive, Per Cent |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | 1:10 | Controls | 143 | 0 | 8 | 4 | 4 | 5 | 3 | 8 | 0 | 100 |
| 7 | 1:15 | Controls | 143 | 0 | 7 | 1 | 6 | 2 | 5 | 5 | 2 | 71 |
| 7 | 1:20 | Controls | 143 | 1 | 6 | 2 | 4 | 2 | 4 | 2 | 4 | 33 |
| 21 | 1:15 | 20 | 150 | 1 | 20 | 7 | 14 | 10 | 11 | 13 | 8 | 61 |
| 24 | 1:15 | Controls | 150 | 1 | 23 | 7 | 17 | 10 | 14 | 14 | 10 | 58 |

emphasized, however, that data are needed from other dosage levels before general conclusions can be drawn on these phases of the problem. The average weight of experimental and control animals at this time was 22.5 Gm. and 23.8 Gm., respectively.

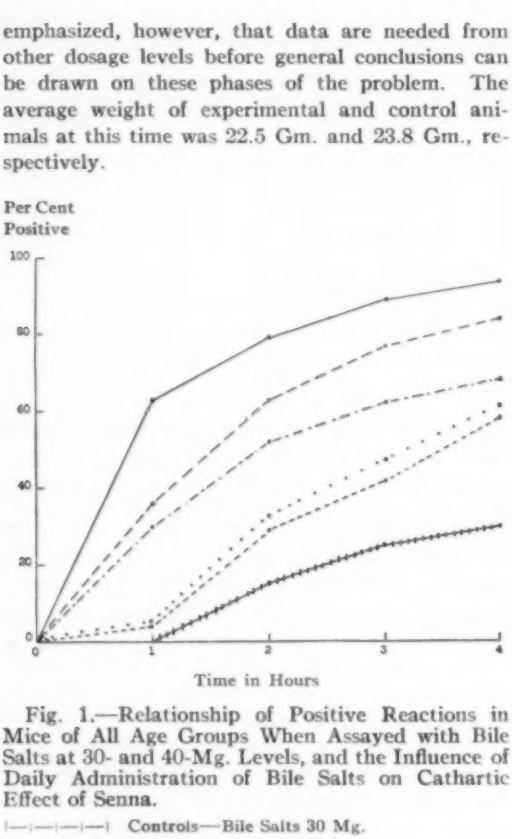



Fig. 1.—Relationship of Positive Reactions in Mice of All Age Groups When Assayed with Bile Salts at 30- and 40-Mg. Levels, and the Influence of Daily Administration of Bile Salts on Cathartic Effect of Senna.

|—|—|—|—| Controls—Bile Salts 30 Mg.
— · — · — · Controls—Bile Salts 40 Mg.
— — — — Bile Salts Daily 20 Mg.—Bile Salts 30 Mg.
———— Bile Salts Daily 20 Mg.—Bile Salts 40 Mg.
- - - - - - Controls—Fldext. Senna, 1:15
. . . . . Bile Salts Daily 20 Mg.—Fldext. Senna, 1:15

Thirty-four days (154 days, total) following the last assay with bile salts, the control mice received 30 mg. of this preparation. The results are shown in Table II. The difference between these results and those obtained from the 90- and 120-day experimental animals at the same dosage levels, and from the 120-day controls at 40 mg., would seem to indicate that the susceptibility of the controls to bile salts has shown a considerable increase. However, the results obtained in a subsequent assay (Fig. 1 and Table II, 166-day controls), in which only 30% of animals were positive at the same dosage level (30 mg.), would seem to discount the possibility of an increased susceptibility to bile salts in the controls. The administration of fluidextract of senna to this group only four days previously may have influenced these results. Further investigation of this phase of the problem is under consideration.

Following a 37-day period (157 days, total), the assay with bile salts was repeated in the experimental animals, using the T. C. D. as the assay dose. The results (Table II) compare favorably with those obtained from the 60-day experimental group which also received 40 mg. as the assay dose. Following this assay daily administration of bile salts was discontinued. Eight days later an assay dose of 30 mg. was given to these animals. The results are shown in Table II. The response was approximately 30% less than when these animals were receiving bile salts daily, and was also less than the average response of the control animals following 40-mg. doses.

The percentage relationship of the positive reactions of control and experimental animals when assayed with bile salts at 30- and 40-mg. levels, is shown in Fig. 1. The influence of time as an important factor in the assay is emphasized since Fig. 1 clearly shows that a comparatively long period of time is required for accurate evaluation of the cathartic action of bile salts by this method.

## DISCUSSION

The results of the experiments presented in this report indicate that mice are desirable animals for the study and evaluation of effects produced by chronic oral administration of cathartic drugs. Based upon the methods described, the acute and chronic cathartic effects of bile salts were investigated.

The animals employed in these studies exhibited no tolerance to threshold doses following chronic daily administration of bile salts. Indeed, it would appear that the susceptibility of mice to threshold doses of the drug is increased inasmuch as the per cent of positive results in treated animals following a 40-mg. assay dose was always greater than in the controls at the same assay dose, and the results from 30-mg. assay doses in the experimental animals were comparable to 40 mg. in the controls. Some

of the more obvious causes of increased susceptibility to the cathartic action of bile salts are:

1. Accumulation of the drug in the body.
2. A difference in weight of the control and experimental animals when a fixed dose is administered upon a weight per mouse basis.
3. A moderate evacuation of the bowels insufficient to cause fluidity of the stools.
4. A mild, repeated, non-specific irritation of the gastro-intestinal mucosa, due to daily application of bile salts.
5. A specific cellular phenomenon by which the susceptibility of the gastro-intestinal tract to bile salt action is increased.
6. A greater combined total of bile constituents in the intestine due to the probable choleretic effect of daily administration of bile salts (6, 8, 9).

Accumulation of the drug in the body is not responsible for the increased susceptibility since daily administration of relatively small doses (2 and 4 mg.) and daily administration of 20-mg. doses of bile salts, representing one-half the T. C. D., induce equivalent increases in suceptibility (Table II). Furthermore, the daily administration of approximately one-half the T. C. D. for a period of 90 days did not induce active catharsis in the animals, indicating that a minimum of 50% of the T. C. D. of bile salts is essentially eliminated within 24 hours. Since an interval of 24 hours was allowed to elapse between the last daily dose and the administration of any assay dose, it is apparent that the acute effects of the last daily dose upon the effect of the assay dose is negligible.

The average weight of both control and experimental animals, which fluctuated between 20 and 24 Gm. throughout the duration of these studies, apparently did not affect the results. This observation has previously been made by Geiger (7), who employed mice weighing between 20 and 24 Gm.

A moderate evacuation of the bowels insufficient to cause fluidity of the stools does not appear to be a causative factor in the production of increased susceptibility to bile salts. If such an action of the drug brought about a more intimate contact of the drug with the intestinal mucosa, the response of threshold doses of senna presumably would be greater in those animals treated with bile salts than in the controls. Figure 1 and Table III indicate that this is not the case.

Evidence cited above would indicate that a mild, repeated, non-specific irritation of the gastro-intestinal mucosa, due to daily application of bile salts, is not produced, since there is no difference in the response of the two groups to senna.

Either or both of the two remaining factors (5 and 6, above) appear to be the mechanisms responsible for the increase in susceptibility to bile salts. Data obtained thus far in this investigation do not distinguish between the two. It is interesting to note that a comparison of the results presented in Tables II and III, and Fig. 1, shows that the increased susceptibility is specific for bile salts to the extent that the approximate T. C. D. of senna is not influenced. From an analysis of data presented in Table II, it appears that increased susceptibility is a factor of continuous application of bile salts inasmuch as after a period of eight days, during which no bile salts were given, the response to the 30-mg. assay dose was appreciably diminished.

## CONCLUSIONS

1. A method for chronic study of cathartic drugs in mice is described.
2. Bile salts in sufficient quantity will induce active catharsis in mice and this action may be evaluated by the method described.
3. The threshold cathartic dose of bile salts for mice was determined to be approximately 40 mg. per mouse. One-half of this dose administered daily for relatively long periods induces no active catharsis.
4. Daily administration of sub-threshold doses of bile salts to mice for a period of about 150 days induces no tolerance to the cathartic effect of the threshold dose of this drug, given 24 hours after the last daily administration.
5. Within the limits of these experiments, it appears that such daily administration induces an increased susceptibility

to the cathartic action of bile salts. The probable causes of this phenomenon and its dependency on daily application are discussed.

6. The results of a single assay, using a 1:15 dilution of fluidextract of senna, indicate that its cathartic effect remains practically unchanged following daily administration of bile salts to mice.

7. Approximately four hours are required to develop maximum cathartic action of bile salts in mice.

8. Bile salts induce catharsis more rapidly than senna under the conditions described.

REFERENCES

(1) Fubini, S., and Luzzati, M., *Moleschotts Untersuchungen zur Naturlehre*, 13 (1888), 378–401. Quoted from Horrall, O. H., "Bile, Its Toxicity and Relation to Disease" (1938), University of Chicago Press, pages 183–184.

(2) Horrall, O. H., "Bile, Its Toxicity and Relation to Disease" (1938), University of Chicago Press, pages 185–188.

(3) Haney, H. F., Roley, W. C., and Cole, P. A., *Am. J. Physiology*, 126 (1939), 82–88.

(4) Van Liere, E. J., and Northup, D. W., *Am. Jour. Digest. Dis.*, 8 (Jan. 1941), 26.

(5) Broun, G. O., McMaster, P. D., and Rous, P., *J. Exper. Med.*, 37 (1923), 699–710.

(6) Whipple, G. H., and Smith, H. P., *J. Biol. Chem.*, 80 (1928), 697.

(7) Geiger, E., JOUR. A. PH. A., 29 (1940), 148.

(8) Schmidt, C. R., Beazell, J. M., Atkinson, A. J., and Ivy, A. C., *Am. Jour. Digest Dis.*, 5 (1938), 613.

(9) Co Tui, F. W., *J. Lab. Clin. Med.*, 19 (1934), 567.

# Book Reviews

*Anhydrous Aluminum Chloride in Organic Chemistry*, by CHARLES ALLEN THOMAS in collaboration with MARY BALUK MOSHIER, HERBERT E. MORRIS and R. W. MOSHIER. xiii + 972 pages. 6 x 9 in. 1941. American Chemical Society Monograph No. 87. New York: Reinhold Publishing Corporation $15.00.

The spectacular achievements in the field of synthetic organic chemistry, exemplified especially by the syntheses of vitamins, hormones, chemotherapeutic agents and rubber substitutes are known to everyone. Less spectacular, and consequently not so well known or appreciated, are the discoveries in the purely academic phases of chemistry; however, without these developments, products such as those which have been mentioned never could have been synthesized.

The applications of aluminum chloride in organic chemistry were initiated and studied quite extensively by the two collaborators, the Frenchman, Friedel, and the American, Crafts, purely as an academic project. Subsequent discoveries by hundreds of other investigators have increased the range of usefulness of aluminum chloride to an astonishing extent. The collection of the vast amount of data in this monograph, and its arrangement under pertinent chapter headings, have made information readily available; the laborious task, performed by the author and collaborators, is one which must be recognized with gratitude not only by the professional chemist, but also by the layman who benefits by the industrial exploitation of chemical syntheses.

This monograph deals not only with Friedel-Crafts syntheses but, as the title indicates, with all types of transformations of organic compounds in which aluminum chloride can play a role.

It is stated in the preface that "Every effort has been made to make it complete and to include all references of published material, both of a purely scientific and industrial nature, including the available patent literature;" these objectives have been realized to an admirable degree. Furthermore, the usefulness of the book has been increased greatly by the inclusion of numerous tabulations of reactions and reaction products and a patent and author index, in addition to the customary subject index.

In view of the obvious importance of the subject, and its excellent and exhaustive treatment, it seems hardly necessary to state that this monograph will be recognized as an indispensable book for every organic chemist.

The binding, printing and paper are of the same fine quality found in the other American Chemical Society Monographs.—F. F. BLICKE